INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, Oct. 1995, p. 670–675 Vol. 45, No. 4
0020-7713/95/$04.00+0

Classification of *Lactobacillus plantarum* by Restriction Endonuclease Analysis of Total Chromosomal DNA Using Conventional Agarose Gel Electrophoresis

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A total of 17 *Lactobacillus plantarum* strains that originated from different environments and 24 reference strains were classified by performing a restriction endonuclease analysis of total chromosomal DNAs digested with *Eco*RI, *Hin*dIII, and *Cla*I, and the resulting patterns were visualized after the fragments were separated according to size by agarose gel electrophoresis. The patterns were analyzed by using the Pearson product moment correlation coefficient and the unweighted pair group algorithm with arithmetic averages. All but two *L. plantarum* strains formed a cluster that was separated from the reference strains at a similarity level of 29% (cluster 1). The two remaining *L. plantarum* strains (cluster 2) merged with cluster 1 at a level of similarity of 28%. The reference strains formed four additional clusters, and four reference strains were stragglers. Cluster 3 (three *Lactobacillus pentosus* strains) and cluster 4 (including *Pediococcus acidilacti* CCUG 32235^T [T = type strain] and *Lactobacillus fermentum* ATCC 14931^T) merged with cluster 1 at a level of similarity of 25%. Cluster 5 comprised 10 *Lactobacillus reuteri* strains, and cluster 6 contained the type strains of *Lactobacillus amylovorus*, *Lactobacillus gasseri*, and *Lactobacillus vaginalis*. Cluster 1 (*L. plantarum*) was divided into three subclusters, and this subdivision reflected to some extent certain phenotypic features of presumed ecological significance, including the ability to adhere to intestinal mucosa (subcluster 1c) and starch and glycogen degradation (subcluster 1a). A principal-component analysis significantly distinguished the strains belonging to different species. Also, the subcluster 1c strains could be separated from the rest of the *L. plantarum* strains. The results of restriction endonuclease analysis of total chromosomal DNA were found to be reproducible, and this method can be used to (i) differentiate between similar strains belonging to the same *Lactobacillus* species and group strains within a species, (ii) distinguish between strains of different *Lactobacillus* species, and (iii) place strains in specific *Lactobacillus* species.

The genus *Lactobacillus* is heterogeneous, and the relationships among the different species have been studied by reverse transcriptase sequencing of 16S rRNAs (2). Larger evolutionary distances between species have been revealed clearly, but the relationships among more closely related species are muddled. Furthermore, rRNA gene sequencing is useless for classification below the species level; for example, the 16S rRNA gene sequences of *Lactobacillus plantarum* and *Lactobacillus pentosus* are identical, and the same is true for the sequences of *Lactobacillus casei* and *Lactobacillus rhamnosus* (2, 12).

Workers have developed a method in which the genomes of *Lactobacillus* strains are characterized by performing a restriction endonuclease analysis (REA) of chromosomal DNAs and then visualizing the resulting fragment patterns after agarose gel electrophoresis is performed (14). This method has been used to classify *Lactobacillus reuteri* strains (13). The general advantage of REA is that it provides a unique genomic fingerprint of the whole chromosomal DNA for each strain. The presence of multiple band patterns, which are obtained by using restriction endonucleases that are able to split the DNA at many sites, means that a taxonomic analysis can be based on a large number of genomic features (DNA fragment sizes) for each strain. At the strain level, the resolution of REA for *L. plantarum* is better than the resolution of ribotyping (6) or randomly amplified polymorphic DNA analysis (7). Furthermore, it has been suggested that this method can identify strains to the species level (13). Thus, REA might supplement 16S rRNA gene sequencing by providing data for the lower taxonomic levels.

L. plantarum is frequently used as a starter culture in lactic acid-fermented foods (9) and has also been isolated from human intestinal mucosa (10). The only previous major taxonomic study of *L. plantarum* at the interspecies level was performed by Dellaglio et al. (3), who divided strains identified phenotypically into two DNA-DNA homology groups. One of these groups was later designated *L. pentosus* (16).

The aims of this study were to use an improved and technically somewhat simplified REA method to classify *L. plantarum* strains isolated from the mucosa of human intestines and from lactic acid-fermented foods and to compare these strains with strains belonging to other species, including the *L. pentosus* type strain and a set of *L. reuteri* strains.

MATERIALS AND METHODS

Strains and culture conditions. The strains which we used are listed in Table 1. All of the *L. plantarum*, *L. pentosus*, and *L. reuteri* strains had previously been genetically identified to the species level by performing DNA-DNA hybridization experiments with the type strains or by ribotyping (6, 13). The strains were grown and stored as described previously (13).

Preparation of DNA and restriction endonuclease digestion. Chromosomal DNAs were prepared after the strains were grown overnight in stationary cultures at 37°C in 100-ml portions of *Lactobacillus* carrying medium (4) containing 1% glucose. Cells were harvested by centrifugation, washed once in 10 ml of TES (50 mM NaCl, 100 mM Tris, 70 mM disodium EDTA; pH 8.0), and suspended in 3 ml of TES supplemented with 25% (wt/vol) sucrose. Then lysozyme (120 mg; grade VI; Sigma Chemical Co., St. Louis, Mo.) and 140 U of mutanolysin (Sigma) were added. The cells were incubated at 42°C for 2 h, 3 mg of proteinase K (type XI; Sigma) was added, and the preparation was incubated at 37°C for 30 min. After cooling, 1 ml of a 5% NaCl solution, 1 ml of 20% sodium dodecyl sulfate, and 11.4 ml of TES were added to the cell suspension. If necessary, the suspension was gently stirred with a glass rod until it became homogeneous, and then it was heated at 65°C for 15 min. The cell lysate was extracted once with

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TABLE 1. Strains used in this study and their sources of isolation

Strain[a]	Source of isolation	Strain no.[b]
Lactobacillus plantarum ATCC 14917^T	Pickled cabbage	1
Lactobacillus plantarum 386[c,d]	Human rectum	2
Lactobacillus plantarum 299[c,d]	Human colon	3
Lactobacillus plantarum 105[c,d]	Human transversum	4
Lactobacillus plantarum 275[c,d]	Human colon	5
Lactobacillus plantarum 299v[c,e]	Sour dough, human intestine	6
Lactobacillus plantarum So5[c,f]		7
Lactobacillus plantarum 256[c,g]	Silage	8
Lactobacillus plantarum 36E[c,h]	Silage	9
Lactobacillus plantarum 79[c,i]	Nigerian pito	10
Lactobacillus plantarum 107[c,i]	Nigerian ogi	11
Lactobacillus plantarum 98[c,i]	Nigerian ogi	12
Lactobacillus plantarum 95[c,i]	Nigerian ogi	13
Lactobacillus plantarum 53[c,i]	Nigerian ogi	14
Lactobacillus plantarum 97[c,i]	Nigerian ogi	15
Lactobacillus plantarum 101[c,i]	Nigerian ogi	16
Lactobacillus plantarum 120[c,i]	Nigerian ogi	17
Lactobacillus plantarum 44[c,i]	Nigerian ogi	18
Lactobacillus plantarum ATCC 8014[c]	Corn silage	19
"*Lactobacillus plantarum*" CH[j,k]		20
"*Lactobacillus plantarum*" 442[l]	Fermented cucumber	21
Lactobacillus pentosus ATCC 8041^T	Corn silage	22
Lactobacillus reuteri DSM 20016^T		23
Lactobacillus reuteri DSM 20015[m]		24
Lactobacillus reuteri N2LC[m]	Rat intestine	25
Lactobacillus reuteri N5LC:2[m]	Rat intestine	26
Lactobacillus reuteri N2J[m]	Rat intestine	27
Lactobacillus reuteri N5D:1[m]	Rat intestine	28
Lactobacillus reuteri 1044[m]	Porcine intestine	29
Lactobacillus reuteri 8557:1[d,m]	Human	30
Lactobacillus reuteri 1048[m]	Porcine intestine	31
Lactobacillus reuteri 8557:3[d,m]	Human	32
Lactobacillus fermentum ATCC 14931^T		
Pediococcus acidilacti CCUG 32235^T		
Lactobacillus gasseri DSM 20243^T		
Lactobacillus vaginalis CCUG 31452^T		
Lactobacillus amylovorus DSM 20532^T		
Lactobacillus helveticus DSM 20075^T		
"*Lactobacillus plantarum*" ATCC 10776[n]		
Erysipelothrix rhusiopathiae[n] CCUG 221^T		
Pediococcus pentosaceus CCUG 32205^T		

[a] ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; CCUG, Culture Collection of the University of Gothenburg, Gothenburg, Sweden.

[b] Strain numbers used in Fig. 3 and 4.

[c] Genetically identified by DNA-DNA hybridization and affiliated with *L. plantarum* (6).

[d] Isolated by Molin et al. (10).

[e] Described by Johansson et al. (5).

[f] Starter culture obtained from the Swedish Sugar Company, Arlöv, Sweden.

[g] Sven Lindgren, Department of Microbiology, Uppsala University, Uppsala, Sweden.

[h] Siv Ahrné, Laboratory of Food Hygiene, Department of Food Technology, Lund University, Lund, Sweden.

[i] Isolated by Johansson et al. (8).

[j] Genetically identified by DNA-DNA hybridization and affiliated with *L. pentosus* (6).

[k] Starter culture obtained from Christian Hansen Laboratories, Copenhagen, Denmark.

[l] H. Fleming, University of North Carolina, Raleigh.

[m] Genetically identified by DNA-DNA hybridization as *L. reuteri* and studied by REA (13).

[n] Genetically identified by DNA-DNA hybridization and found to belong to neither *L. plantarum* nor *L. pentosus* (6).

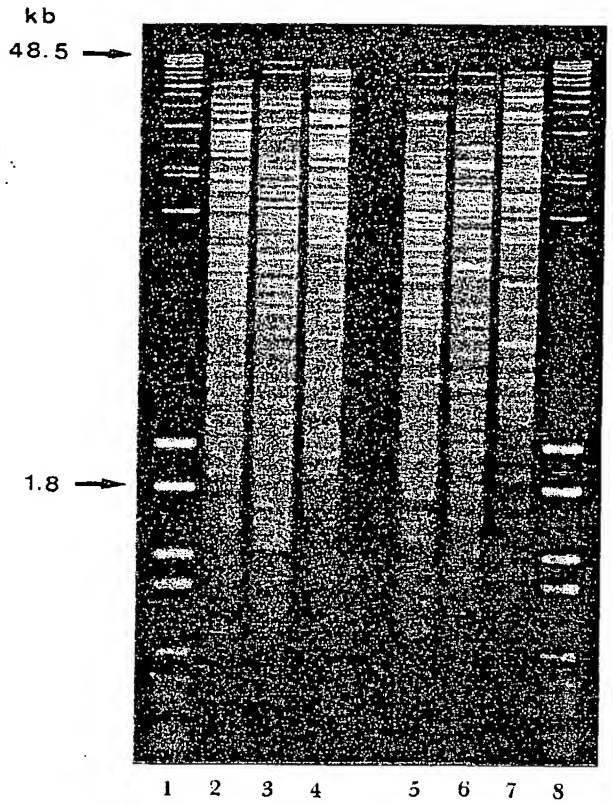

FIG. 1. Agarose gel electrophoresis of *L. plantarum* ATCC 8014 (lanes 2 through 4) and "*L. plantarum*" ATCC 10776 (lanes 5 through 7) DNAs cleaved with *Hin*dIII (lanes 2 and 5), *Cla*I (lanes 3 and 6), and *Eco*RI (lanes 4 and 7). Lanes 1 and 8 contained a high-molecular-weight DNA marker (Bethesda Research Laboratories) and DNA molecular weight marker VI (Boehringer Mannheim). The bands between the arrows were compared.

redistilled phenol-chloroform (1:1) and once with chloroform-isoamyl alcohol (24:1). The DNA was then precipitated with ethanol at −20°C.

The chromosomal DNA was separated from covalently closed circular plasmid DNA (most of the plasmid DNA) by dye buoyant density centrifugation in a CsCl gradient containing ethidium bromide (1). Some linear and open circular forms of plasmid DNA were still present in the preparation, but they contributed very little to the final results (13) (data not shown). The concentration of chromosomal DNA was monitored with a fluorometer (model TKO 100; Hoefer Scientific Instruments, San Francisco, Calif.).

DNA (0.75 μg) was separately digested at 37°C for 4 h with 10 U of *Hin*dIII, *Cla*I, or *Eco*RI (Boehringer Mannheim). Ståhl et al. (14) and Ståhl and Molin (13) used *Asp* 718 instead of *Hin*dIII, but digestion with *Asp* 718 resulted in too many large bands to be optimal for the method which we used.

Electrophoresis and scanning of gels. Gel electrophoresis was carried out by using submerged horizontal 0.9% agarose (ultrapure DNA grade; low electroendoosmosis; Bio-Rad Laboratories, Richmond, Calif.) slab gels (150 by 235 mm). A high-molecular-weight DNA marker (0.2 μg) containing DNA fragments having molecular sizes ranging from 8.5 to 48.5 kb (Bethesda Research Laboratories) and 0.5 μg of DNA molecular weight marker VI containing DNA fragments having molecular sizes ranging from 0.2 to 2.2 kb (Boehringer Mannheim) were used as standards. In addition, pHC 79 digested with *Eco*RI (molecular size, 6.4 kb; Boehringer Mannheim) was added to each well as an internal standard. Minimal band distortion and maximal sharpness were achieved by applying the sample DNA in Ficoll loading buffer (2 g of Ficoll, 8 ml of water, 0.25% bromphenol).

Gels were electrophored at a constant voltage of 40 V for 18 h at 8.5°C; the buffer (89 mM Tris, 23 mM H_3PO_4, 2 mM sodium EDTA; pH 8.3) was recirculated during electrophoresis. Then the gels were stained for 20 min in ethidium bromide (2 μg/ml), destained in distilled water, visualized at 302 nm with a UV

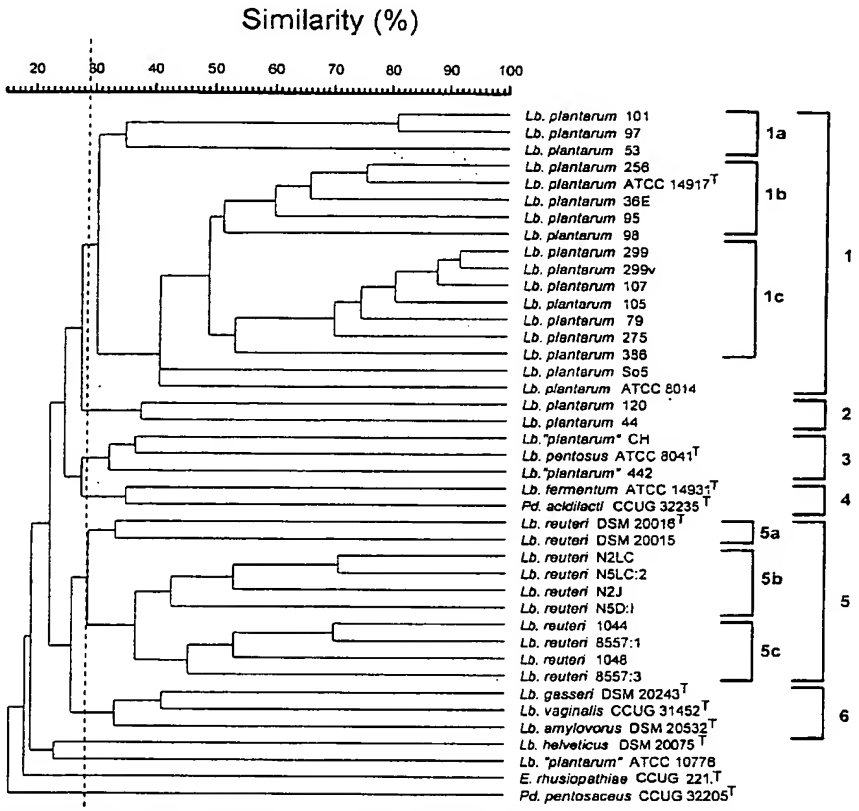

FIG. 2. Dendrogram of the strains tested based on Pearson product moment correlation coefficients and UPGMA clustering. *Lb.*, *Lactobacillus*; *Pd.*, *Pediococcus*; *E.*, *Erysipelothrix*.

transilluminator (UVP, Inc., San Gabriel, Calif.), and photographed. This method of performing gel electrophoresis gave well-distributed and relatively well-separated bands down to a molecular size of about 1.8 kb (Fig. 1).

Reading band patterns. Band patterns on photographic negatives were scanned with a laser densitometer (UltroScan XL; LKB-Produkter AB, Bromma, Sweden). The level of resolution was 1,000 points per lane, and each lane was about 50 mm long. Data were obtained by using the LKB 2400 GelScan XL software package (LKB-Produkter AB). The band patterns were normalized, and background noise was subtracted by using the rolling-disc algorithm of the GelCompar 3.0 program (Applied Maths, Kortrijk, Belgium). The data from the three experiments (the experiments performed with *Hin*dIII, *Cla*I, and *Eco*RI) were combined for each strain by using GelCompar 3.0.

Mathematical analyses. The data set was analyzed by using the Pearson product moment correlation coefficient and the unweighted pair group algorithm with arithmetic averages (UPGMA) (11). The Pearson correlation coefficients and the UPGMA values were calculated for the complete data set, and the GelCompar 3.0 program was used to do this. Our analysis also involved a principal-component analysis (PCA) performed with the strains affiliated with *L. plantarum*, *L. reuteri*, and *L. pentosus*, as well as the *L. plantarum* strains alone (using GelCompar 3.0).

Reproducibility. DNAs from the following strains were analyzed in duplicate (the chromosomal DNA of each strain was prepared twice, and the samples were electrophoresed on different gels): *L. plantarum* 53, 97, ATCC 14917^T (T = type strain), 36E, ATCC 8014, 299, 105, 275, and 120. This analysis was followed by a cluster analysis.

Phenotypic analysis. All *L. plantarum* strains and *L. pentosus* ATCC 8041^T were analyzed by using API 50CH kits (API System, Montalieu, Vercieu, France) as described previously (10) at both 30 and 37°C. The profiles obtained at the two temperatures were combined for each strain (in all, 98 characters were examined) and were compared by using the Jaccard coefficient followed by UPGMA clustering.

RESULTS

Reproducibility. The selected strains analyzed in duplicate merged in the cluster analysis, with no strains in between. This indicated that most of the strains in this study could be distinguished by the REA method which we used. In almost all cases the levels of similarity obtained for duplicate runs of the same strain were in the range from of 90 to 95%.

Cluster analysis. The cluster analysis in which we used the Pearson product moment correlation coefficient followed by UPGMA resulted in the dendrogram shown in Fig. 2. Five clusters were defined at a similarity level of 29%. Cluster 1 (*L. plantarum*) comprised 17 *L. plantarum* strains that originated from both human intestines and fermented foods and feeds, including *L. plantarum* ATCC 14917^T and ATCC 8014. Three distinct subclusters were also identified, and these subclusters merged at similarity levels of 54, 52, and 35% (Fig. 2). *L. plantarum* So5 and ATCC 8014 appeared to be stragglers within cluster 1, merging with the other strains at a similarity level of 42%.

Cluster 2 comprised two *L. plantarum* strains. These strains merged with cluster 1 at a similarity level of 28% (Fig. 2).

Cluster 3 (*L. pentosus*) contained one isolate from pickled cucumber, one commercial starter culture, and *L. pentosus* ATCC 8041^T (Fig. 2).

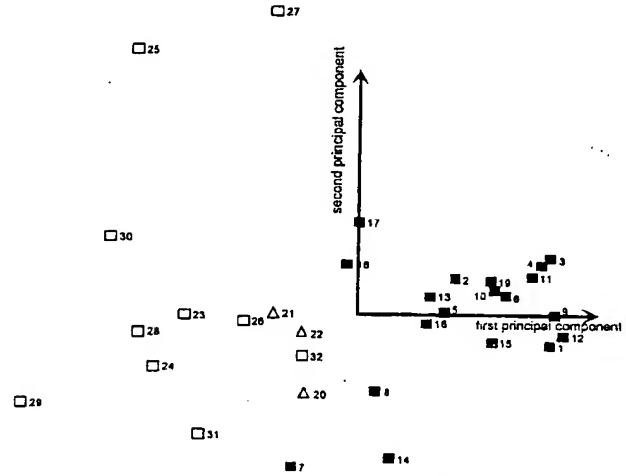

FIG. 3. First- and second-principal-component scores for *L. plantarum* (■), *L. reuteri* (□), and *L. pentosus* (△) strains. The projection was based on the complete data set. The numbers are strain numbers (Table 1).

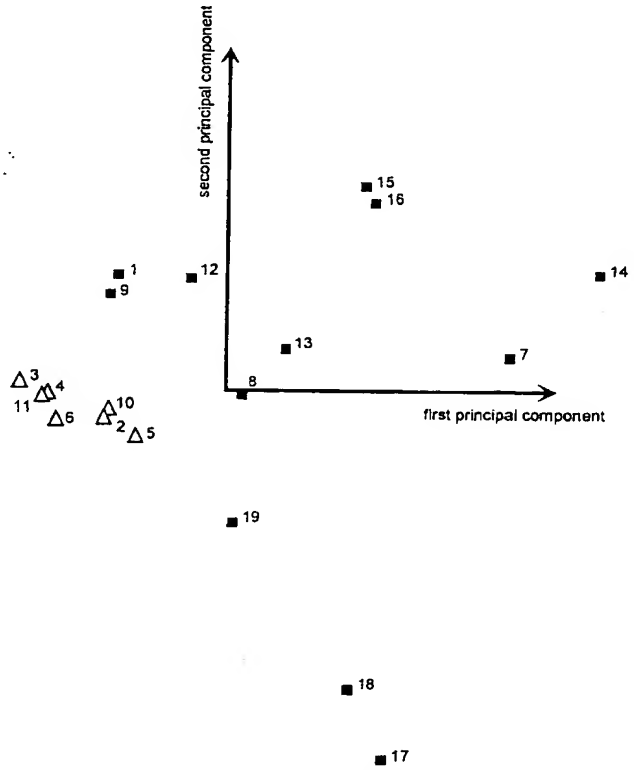

FIG. 4. First- and second-principal-component scores for *L. plantarum* strains, based on the complete data set. Symbols: △, *L. plantarum* strains belonging to subcluster 1c; ■, other *L. plantarum* strains. The numbers are strain numbers (Table 1).

Cluster 4 included two type strains, *Pediococcus acidilacti* CCUG 32235 and *Lactobacillus fermentum* ATCC 14931 (Fig. 2).

Cluster 5 (*L. reuteri*) comprised 10 *L. reuteri* strains, including *L. reuteri* DSM 20016^T and DSM 20015. Cluster 5 could be divided into three subclusters, subclusters 5a through 5c, which merged at similarity levels of 46, 43, and 34%, respectively (Fig. 2). Subcluster 5b contained strains isolated from rat intestines, and subcluster 5c included strains that originated from humans and pigs (Fig. 2). *L. reuteri* DSM 20016^T and DSM 20015 (subcluster 5a) merged with cluster 5, but at a lower level of similarity (29%).

A sixth cluster, cluster 6, contained three type strains, *Lactobacillus gasseri* DSM 20243, *Lactobacillus vaginalis* CCUG 31452, and *Lactobacillus amylovorus* DSM 20532 (Fig. 2).

Three type strains and one reference strain formed one-member clusters (Fig. 2).

PCA. The PCA and accompanying statistical evaluation of *L. plantarum*, *L. pentosus*, and *L. reuteri* strains revealed that there were three groups (coinciding with the species affiliations) that were significantly separated from each other (Fig. 3) (all data are not shown). Strains that were located peripherally in each group were removed and tested individually to determine their group affiliations. This analysis showed that each strain was more closely related to its assigned species than to any of the other species (data not shown). The *L. plantarum* group of strains (clusters 1 and 2) was more compact than the *L. reuteri* group of strains (Fig. 3) (data not shown).

A separate PCA was performed with the *L. plantarum* strains, and the strains in subcluster 1c formed a compact group that was separated from most of the other strains by both the first and second principal components (Fig. 4). It is also noteworthy that the strains in subcluster 1b (Fig. 2) are the strains that merged closest to cluster 1c in the PCA. According to the PCA plot, *L. plantarum* So5 and 53 are the strains that are most distantly related to subcluster 1c according to the first principal component, while *L. plantarum* 44 and 120 are the strains that are most distantly related according to the second principal component.

Phenotype. We compared the abilities of the different *L. plantarum* strains (and *L. pentosus* ATCC 8041^T) to ferment different carbohydrates (as determined by API 50CH tests), and the resulting dendrogram is shown in Fig. 5. The clustering in this dendrogram resembles in several respects the clustering obtained with the REA. However, strain ATCC 10776 was misidentified by API 50CH tests as a *L. plantarum* strain, and the phenotypic tests failed to group the strains in subcluster 1c in accordance with their genomic relationships.

DISCUSSION

The REA method described by Ståhl et al. (14) and Ståhl and Molin (13) was modified in this study by changing one of the restriction enzymes (*Asp* 718 to *Hin*dIII), shortening the running time of the agarose gels, and utilizing the GelCompar program instead of SIMCA for evaluation. These modifications made the REA easier to handle and allowed us to use a larger proportion of the DNA fragments; because of the long time that elapsed at the original setting, fragments that were smaller than 6.4 kb ran out of the gels. Thus, in this study almost the entire chromosomal DNA was used for to compare strains.

In order to evaluate whether our results were comparable to the results obtained by Ståhl and Molin (13), a set of *L. reuteri* strains that previously had been classified by REA (13) was included in the study. In this study, these strains formed a separate cluster at a similarity level of 29% (cluster 5). The

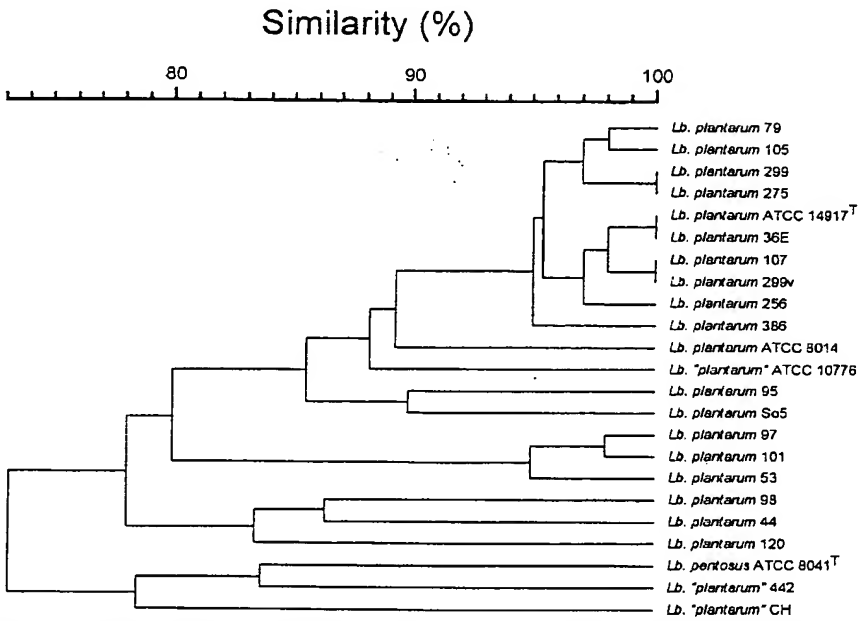

FIG. 5. Dendrogram based on an analysis of combined API 50CH profiles (obtained at both 30 and 37°C) followed by an analysis in which the Jaccard coefficient and UPGMA were used, showing the phenotypic relationships among the *L. plantarum* and *L. pentosus* strains included in this study. *Lb.*, *Lactobacillus*.

formation of subclusters and the relationships among several strains within the subclusters were consistent with the results reported by Ståhl and Molin (13), and this was true despite the fact that one of the restriction enzymes was changed.

All of the *L. plantarum* strains included in cluster 1 have been shown previously either to exhibit high levels of DNA-DNA homology with the type strain of *L. plantarum* or to have the same ribopatterns (as determined with *Eco*RI- and *Hin*dIII-cleaved DNA) as *L. plantarum* ATCC 14917^T (6). Interestingly, *L. plantarum* 44 and 120, both of which merge with cluster 1 at a similarity level of 28%, were found to have different ribopatterns and to exhibit relatively low levels of DNA-DNA homology with the type strain of *L. plantarum* (6). "*L. plantarum*" ATCC 10776 was singled out from the rest of the *L. plantarum* strains by REA (Fig. 1). This is consistent with the DNA-DNA homology data obtained with the type strain of *L. plantarum* (3, 6). However, on the basis of the results of phenotypic tests "*L. plantarum*" ATCC 10776 can be identified as a member of the species (Fig. 5).

Three distinct subclusters were identified within cluster 1, but the groups did not reflect the sources of isolation. However, we identified some common phenotypic characteristics within subclusters 1a and 1c. Subcluster 1a was made up of three strains that are able to ferment both soluble starch and glycogen, and subcluster 1c (also determined to be a group of closely related strains by PCA) included strains that have the ability to adhere in vitro to human intestinal cell line HT-29 via an α-methyl-mannoside-inhibited binding mechanism (15). Two of the strains in subcluster 1c (*L. plantarum* 299 and 299v) have also been shown to be able to colonize human intestinal mucosa in vivo after oral administration (5). The similarities between strains as well as subclusters of *L. plantarum* were supported by the results obtained by the PCA. We found that phenotypic tests could not be used to group the strains in subcluster 1c (Fig. 5).

REA data can be used to distinguish between very closely related strains. To our knowledge, there are few methods available that are able to identify strains down to such a low level. However, proper gel conditions are necessary i.e., the amount of DNA applied has to be the same, and dots and dirt in the lanes of the gel, as well as normalization errors, must be avoided.

The REA method described in this paper, combined with UPGMA or PCA, has potential for classifying strains at the species level and below. An interesting question is whether this method can be used to estimate levels of relatedness between closely related species. The low levels of similarity between the most distantly related strains belonging to the same species which we observed argue against this possibility. On the other hand, the *L. pentosus* cluster appeared to be closer to the *L. plantarum* clusters than the *L. reuteri* strains did (Fig. 2). However, to answer this question, the data set has to be expanded, and the REA results have to be compared with results obtained by using available methods for phylogenetic studies.

ACKNOWLEDGMENTS

We thank Marie Kala for her skillful technical assistance.

This work was supported by the Swedish Agency for Research Cooperation with Developing Countries (SAREC) and the Swedish Work Environment Fund.

REFERENCES

1. **Bauer, W., and J. Vinograd.** 1968. The interaction of closed circular DNA with intercalative dyes. I. The superhelix density of SV 40 DNA in the presence and absence of dye. J. Mol. Biol. 33:141–171.
2. **Collins, M. D., U. Rodrigues, C. Ash, M. Aguirre, J. A. E. Farrow, A. Martinez-Murcia, B. A. Phillips, A. M. Williams, and S. Wallbanks.** 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol. Lett. 77:5–12.
3. **Dellaglio, F., V. Bottazi, and M. Vescovo.** 1975. Deoxyribonucleic acid homology among *Lactobacillus* species of the subgenus *Streptobacterium* Orla Jensen. Int. J. Syst. Bacteriol. 25:160–172.

4. **Efthymiu, C., and P. A. Hansen.** 1962. An antigenic analysis of *Lactobacillus acidophilus*. J. Infect. Dis. 110:258–267.
5. **Johansson, M.-L., G. Molin, B. Jeppsson, S. Nobaek, S. Ahrné, and S. Bengmark.** 1993. Administration of different *Lactobacillus* strains in fermented oatmeal soup: in vivo colonization of human intestinal mucosa and effect on the indigenous flora. Appl. Environ. Microbiol. 59:15–20.
6. **Johansson, M.-L., G. Molin, B. Pettersson, M. Uhlén, and S. Ahrné.** Characterization and species recognition of *Lactobacillus plantarum* strains by restriction fragment length polymorphism (RFLP) of the 16S rRNA gene. J. Appl. Bacteriol., in press.
7. **Johansson, M.-L., M. Quednau, G. Molin, and S. Ahrné.** Randomly amplified polymorphic DNA for rapid typing of *Lactobacillus plantarum* strains. Lett. Appl. Microbiol., in press.
8. **Johansson, M.-L., A. I. Sanni, C. Lönner, and G. Molin.** 1995. Phenotypically based taxonomy using API 50CH, of lactobacilli from Nigerian ogi, and the occurrence of starch fermenting strains. Int. J. Food Microbiol. 25:159–168.
9. **McKay, L. L., and K. A. Baldwin.** 1990. Applications for biotechnology: present and future improvements in lactic acid bacteria. FEMS Microbiol. Rev. 87:3–14.
10. **Molin, G., B. Jeppsson, M.-L. Johansson, S. Ahrné, S. Nobaek, M. Ståhl, and S. Bengmark.** 1993. Numerical taxonomy of *Lactobacillus* spp. associated with healthy and diseased mucosa of the human intestines. J. Appl. Bacteriol. 74:314–323.
11. **Romersburg, H. C.** 1984. Cluster analysis for research. Lifetime Learning Publications, Belmont, Calif.
12. **Schleifer, K. H., W. Ludwig, R. Amann, C. Hertel, M. Ehrmann, W. Köhler, and A. Krause.** 1992. Phylogenetic relationships of lactic acid bacteria and their identification with nucleic acid probes, p. 23–32. *In* Les Bactéries Lactiques. Adria Normandie, Centre de Publications de l'Université de Caen, Caen, France.
13. **Ståhl, M., and G. Molin.** 1994. Classification of *Lactobacillus reuteri* by restriction endonuclease analysis of chromosomal DNA. Int. J. Syst. Bacteriol. 44:9–14.
14. **Ståhl, M., G. Molin, A. Persson, S. Ahrné, and S. Ståhl.** 1990. Restriction endonuclease patterns and multivariate analysis as a classification tool for *Lactobacillus* spp. Int. J. Syst. Bacteriol. 40:189–193.
15. **Wold, A. (Department of Clinical Immunology, University of Gothenburg, Gothenburg, Sweden).** 1995. Personal communication.
16. **Zanoni, P., J. A. E. Farrow, B. A. Phillips, and M. D. Collins.** 1987. *Lactobacillus pentosus* (Fred, Peterson, and Anderson) sp. nov., nom. rev. Int. J. Syst. Bacteriol. 37:339–341.

DEVELOPMENT OF SAFE ORAL VACCINES BASED ON *LACTOBACILLUS* AS A VECTOR.

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in: Recombinant Vaccines: New Vaccinology (ed. E. Kurstak),
Int. Comp. Virology Org., Montreal, 1993, in press.

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INTRODUCTION

Under the declaration of "Children's Vaccine Initiative" and the "Transdisease Vaccinology program" (WHO/UNDP) a number of prerequisites for a new vaccine to be used for universal immunisation were phrased (see also: Bloom 1989). Ideally these vaccines should be given orally in a single dose (1-2, not multiple) early in life as a cocktail for a variety of diseases. Furthermore, they should be safe, cheap, stable and easy to administer (cf. Mestecky 1987). The scientific consensus at this point is that live vaccines are potential candidates for single oral immunisation (Bloom 1989; Brown 1989). These vaccines consist of a microorganism as vector (bacteria or virus) which contains "foreign" genetic material able to induce neutralising immune responses against selected pathogens. In this way the proteins produced by the carrier will induce a protective immune reponse in the host, without the need for actual contact with the pathogen itself. A major advantage being the fact that these vectors are delivered and induce immunity at the mucosal surface, the site where the infection actually occurs and the first line of defence (McGhee et al. 1992). Although some succes has been obtained a disadvantage until now has been the fact that the carriers used (e.g. *Salmonella, E. coli,* Vaccinia) can not be classified as "safe". Furthermore these carriers are highly immunogenic by themselves, thereby drawing unnecessary attention of the immune system and maybe even hampering repetitive use of the vaccine carrier with other antigens.

In vaccination programs in which large numbers of subjects are involved, the oral route of administration is a most convenient route of vaccination as compared to the more frequently used parenteral routes. Mainly because of the less stringent criteria for a) application (feeding vs injection), b) education of trained healthcare workers and c) the induction of both mucosal and systemic immuneresponses. For the above and other reasons the use of a GRAS (Generally Recognized As Safe) organism with probiotic properties, such as *Lactobacillus*, as a safe vector for the delivery of foreign antigens in the gastrointestinal tracts of animals and humans could be preferred.

LACTOBACILLUS

The genus *Lactobacillus* belongs to the lactic acid bacteria, a group of microorganisms that have been used since time immemorial in the preparation and processing of food and beverages. These rod-shaped, non-pathogenic bacteria are nowadays used in numerous fermentation processes as starters, either alone or in combination with other microorganisms such as streptococci, leuconostocs, pediococci and yeasts (Rose 1982; Chassy 1985; Table 1). Since a number of years lactobacilli also find application in the animal food industry, for the valorization and conservation of feed. Lactobacilli are capable of rapidly converting the available carbohydrates into lactic acid which lowers the pH, thus preventing the outgrowth of undesirable pathogenic microorganisms. A second useful property of certain *Lactobacillus* strains in this respect is their capacity to degrade plantwaste material containing polysaccharides like starch and fructan, yielding products that can be more rapidly digested by animals. Based on Metchnikoff's theory that harmful effects of undesired bacteria can be overcome by establishing a new balance

between intestinal bacteria, through ingestion of lactobacilli or products made by these organisms, some strains of lactobacilli are believed to have certain health promoting activities (Metchnikoff 1908). These assumed beneficial effects of lactobacilli have attracted the interest of numerous investigators both from academia and from industry, to explore the potential usefulness of this group of bacteria as probiotics. Research carried out during the last few decades suggests that lactobacilli may indeed beneficially affect humans and animals in various ways (Table 2). Alleged health and/or nutritional benefits for humans and animals associated with the consumption of fermented milk products include: control of intestinal infections, improved nutritional value of some foods, control of serum cholesterol levels, improvement of lactose metabolism, induction of a-specific and specific immune responses and anticarcinogenic activity (Fernandes et al 1987; Gurr 1987; Perdigon et al 1988; Gilliland 1990; Gerritse et al, 1991a,b). Together, these findings may have important implications for the development of novel, *Lactobacillus*-based, oral vaccines.

Table 1 INDUSTRIAL APPLICATIONS OF *LACTOBACILLUS* STRAINS

Product	Organisms used
Yoghurt	*L. bulgaricus, Streptococcus thermophilus*
Fermented milks	*L. acidophilus, L. casei, L. bifidus, L. bulgaricus*
Cheeses	*L. bulgaricus, L. helveticus*
Soy sauce	*L. delbrueckii*
Sour bread	*L. sanfrancisco*
Crackers	*L. plantarum*
Sauerkraut	*L. plantarum, L. brevis*
Cucumbers (pickles)	*L. plantarum*
Green olives	*Lactobacillus* spp.
Cured ham	*L. casei, L. plantarum*
Sausages, meats	*L. plantarum, L. reuteri,* other spp., *Pediococcus* spp.
Distillery mashes	*L. casei, L. fermentum, L. plantarum, L. delbrueckii*
Feed additives	*L. acidophilus, L. bulgaricus, L. lactis*
Silage starters	*L. plantarum, Pediococcus* spp.
Lactic acid	*L. delbrueckii*

LACTOBACILLUS AS A VECTOR FOR ORAL IMMUNIZATION ?

As stated above, *Lactobacillus* is widely known for its capacity to ferment food components, for its supposed health promoting (probiotic) properties and as a normal constituent of the human gut flora. These factors make *Lactobacillus* a very attractive candidate for use as an oral vaccine. Although not the purpose of our experiments, protein malnutrition and vitamin deficiencies could also be, at least partially, battled with the aid of *Lactobacillus* fermented food. The use of *Lactobacillus* over other microorganisms offers some major advantages, 1) *Lactobacillus* is a GRAS (generally recognized as safe) organism, 2) *Lactobacillus* possesses intrinsic adjuvanticity 3) *Lactobacillus* is a "normal" commensal of the gut, and 4) *Lactobacillus* has several additional probiotic effects (it should be taken into account that the underlying mechanisms of

APPLICATIONS OF *LACTOBACILLUS* IN PUBLIC AND ANIMAL HEALTH

- Improvement of the nutritive value of food
- Antimicrobial activity
- Reduction of serum cholesterol levels
- Immunoadjuving effect
- Oral immunisation
- Detoxification of carcinogens

these probiotic effects have not all been fuly explained or confirmed). To maximize the effect that any combination of these properties can have on the induction of a protective immune reponse against e.g. a virus from which a neutralizing epitope is expressed by *Lactobacillus*, we will attempt to answer the following experimental questions: Should an antigen, expressed by *Lactobacillus*, remain intracellular (protection from degradation), be secreted (only use adjuvanticity of L.) or surface bound (carrier effect of L.) to induce an optimal protective immune response? Can effective humoral and cellular immunity be obtained with live (vs killed) Lactobacilli, and is colonization an advantage (persistence of protection) or a disadvantage (induction of tolerance)?

In order to be able to fully exploit the great potential of lactobacilli as probiotics, in particular as oral vaccines, an understanding of the mechanisms underlying the aforementioned phenomena is a prerequisite. Such an understanding requires an analysis at the molecular level of processes like colonization of the intestinal mucosa, stimulation of the immune response, alteration of bile-acid metabolism etc, as these processes are thought to play a major role in the probiotic effects of lactobacilli. In addition, detailed knowledge of the mechanisms controlling gene expression has to be gathered, if one ever wants to control such processes. The application of recombinant DNA techniques has already proven to be very valuable in the analysis of such processes. In the next paragraphs the state of the art of gene manipulation in lactobacilli will be described after this a summary of immunological studies performed with *Lactobacillus* will be given.

GENE-TRANSFER SYSTEMS

In order to develop *Lactobacillus* as a safe (non-pathogenic) vector for the delivery of antigens in the gastrointestinal tract, methods need to be available which allow the introduction (and subsequent expression) of foreign antigens in *Lactobacillus* strains of choice.

Since the onset of recombinant DNA research, systems for genetic manipulation have been developed for several industrially important organisms like *Bacillus*, *Streptomyces* and *Lactococcus*. Based on the knowledge generated with these organisms, rapid progress has recently been made in the development of gene-transfer and gene-expression systems for lactobacilli. Aided by the development of a reproducible system to transform *Lactobacillus* strains (Chassy & Flickinger 1987; Aukrust & Nes 1988; Luchansky et al 1988), methods have become available to (re)introduce DNA into these organisms. The efficiency of transformation of lactobacilli, which

is based on electroporation of the bacteria, is strain dependent. Whereas strains from species like *L.casei*, *L.plantarum* and *L.reuteri* can be transformed with high efficiency (10^5-10^7 CFU/μg DNA), others can be transformed with very low efficiency only, or not at all (Posno et al 1991b; Ahrné et al 1992; Leer et al 1992). In particular, strains originating from the gastro-intestinal tract of animals and humans as *e.g. L.acidophilus* strains, and strains used in dairy industry such as *L. delbruekii* subsp. *bulgaricus* and *L.helveticus* are refractory to efficient transformation.

To date a spectrum of plasmid vectors with *Lactobacillus* replicons has been constructed, allowing genetic manipulation of a wide variety of *Lactobacillus* species, including species that can colonize the gastro-intestinal tract of humans and animals. Most plasmid vectors are derived from small cryptic plasmids of different *Lactobacillus* species. Such plasmids replicate through a mechanism of rolling-circle-replication (Gruss & Ehrlich 1989). This may affect the stability of recombinant plasmids, as will be shown in a subsequent section. Table 3 presents a list of plasmid

Table 3 PLASMID VECTORS WITH *LACTOBACILLUS* REPLICON

Plasmid	Replicon	Origin	*E.coli* DNA	Size (kb)	Marker	Cloning sites	References
pLE16	pLB10	*L.bulgaricus*	pBR328	7.6	*cml*	*Hin*dIII	Chagneaud et al 1992
pBG10	pLJ1	*L.helveticus*	pBR329	6.0	*lacZ*	*Bam*HI,*Pst*I	Hashiba et al 1992
pLP3537	p353-2	*L.pentosus*	pUC19	6.3	*ery*	*Eco*RI,*Hin*dIII	Posno et al 1991a
pLP317	p353-1	*L.pentosus*	no	2.9	*ery*	(*Sph*I)	Posno et al 1991a
pLP317*cop*	p353-1	*L.pentosus*	no	2.9	*ery*	(*Sph*I)	Posno et al 1991a
pLPE323	p353-2	*L.pentosus*	no	3.6	*ery*	*Eco*RI,*Xba*I	Posno et al 1991a
pLPE350	p353-2	*L.pentosus*	no	3.9	*ery*	*Hin*dIII,*Kpn*I,*Pst*I *Sma*I, *Sph*I	Leer et al 1992
pLPE23M	p353-2	*L.pentosus*	no	3.7	*ery*	MCS*	Pouwels & Leer 1993
pLPE24M*cop*	p353-2	*L.pentosus*	no	3.7	*ery*	MCS	Pouwels & Leer 1993
pLP3537*xyl*	p353-2	*L.pentosus*	pUC	6.3	*xyl,ery*	*Xba*I	Posno et al 1991b
pLP825	p8014-2	*L.plantarum*	pBR322	7.7	*cml*	*Acc*I,*Sal*I,*Sph*I	Leer et al 1987; Posno et al 1991a
pLP82H	p8014-2	*L.plantarum*	pBR322	7.7	*ery*	*Bam*HI,*Sal*I,*Sph*I	Posno et al 1991a
pLPC37	p8014-2	*L.plantarum*	no	3.7	*cml*	*Eco*RV,*Sph*I	Leer et al 1992
pULP8/9	pLP1	*L.plantarum*	pUC	6.6	*ery*	*Hin*dIII	Bouia et al 1989
pSC10		*L.plantarum*	no	3.0	*ery*	*Bcl*I	Cocconcelli et al 1991
pPSC20		*L.plantarum*	no	5.5	*cml, ery*	*Bcl*I	Cocconcelli et al 1991
pPSC22		*L.plantarum*	no	4.3	*cml, ery*	*Bcl*I	Cocconcelli et al 1991
pLUL634	pLUL631	*L.reuteri*	no	5.1	*ery*	*Cla*I, *Hpa*I	Ahrné et al 1992
pTRKH2	pAMβ1	*E.faecalis*	p15A	6.9	*cml,ery*	MCS	O'Sullivan & Klaenhammer 1993
pTRKL2	pAMβ1	*E.faecalis*	p15A	6.4	*ery*	MCS	O'Sullivan & Klaenhammer 1993

* MCS, multi-cloning site

vectors currently in use in various laboratories.

Most vectors, as for example pLP825 and pLPE323, display a wide-host-range phenotype, since they can be propagated in a wide variety of *Lactobacillus* species (Posno et al 1991b). Moreover, their copy number in different *Lactobacillus* strains does not significantly differ, indica-

ting that also control mechanisms for DNA replication in different host bacteria operate in a similar way. The average copy number of these plasmid vectors is estimated at 30-50 copies per cell. An improved version of vector pLPE323, named pLPE23M was obtained by introduction of a multi-linker region with 19 unique restriction enzyme sites. The usefulness of the vector has been demonstrated by cloning and overexpression of several genes in *Lactobacillus*.

Recently, a series of wide-host-range vectors was constructed for *Lactococcus* based on the replication functions of the theta-type plasmid pAMβ1 (O'Sullivan & Klaenhammer 1993). They might be useful for cloning in *Lactobacillus* as plasmids replicating by a theta-type mechanism show structural and segregational stability (Swinfield et al 1991; Brückner 1992). Also a vector with narrow host-range has been described. Plasmid pLUL631 from *L.reuteri* carrying a erythromycin-resistance gene was found to replicate only in *L.reuteri* and in a strain of *L.fermentum* among several lactobacilli and other Gram-positive bacteria tested (Ahrné et al 1992). Similarly, a 4.4 kb plasmid replicon from *L.crispatus* was found to replicate only in the host strain from which it was derived (Posno, unpublished observations). The latter type of vectors offers attractive properties with regard to safety aspects associated with the use of live recombinant DNA organisms in *e.g.* food products. Vectors with a narrow host-range are less likely to be horizontally transferred to other bacterial species than vectors based on wide-host-range replicons, and are, consequently, intrinsically more safe.

STRUCTURAL STABILITY

For any application, either in industrial fermentation processes or as oral vaccine, it is essential that the vector remains structurally intact (structural stability) and can be stably maintained in the host cell in the absence of selective pressure (segregational stability). No structural instability was observed in different *Lactobacillus* strains after insertion into *Lactobacillus* vectors of DNA fragments originating from either *Lactobacillus* or other sources (Posno et al 1991a; Leer et al 1992). It appears that except for cases where expression of the cloned gene results in a deleterious protein, cloning of homologous and heterologous DNA into *Lactobacillus* offers no serious problems. Even relatively large fragments can be stably maintained without the occurrence of detectable deletions or rearrangements.

SEGREGATIONAL STABILITY

Most vectors are rapidly lost (50 -> 95% loss after 100 generations) when lactobacilli are cultivated in the absence of the selective agent (Bringel et al 1989; Posno et al 1991b; Szimizu-Kadota et al 1991). Vectors with a replicon from *Lactococcus* or *Staphylococcus* are even less stable in *Lactobacillus*, showing segregation rates of several percent per generation (Posno et al 1991b; Szimizu-Kadota et al 1991). Some vectors with *Lactobacillus* replicons can, however, be stably maintained for more than 100 generations in *Lactobacillus* in the absence of selective pressure (Posno et al 1991b; Cocconcelli et al 1991; Leer et al 1992). Of particular interest is plasmid pLPE323 which was found to be segregationally fully stable in all except one *Lactobacillus* strain (Leer et al 1992; Fig.1). Plasmid pLP3537, which is rapidly lost under non-

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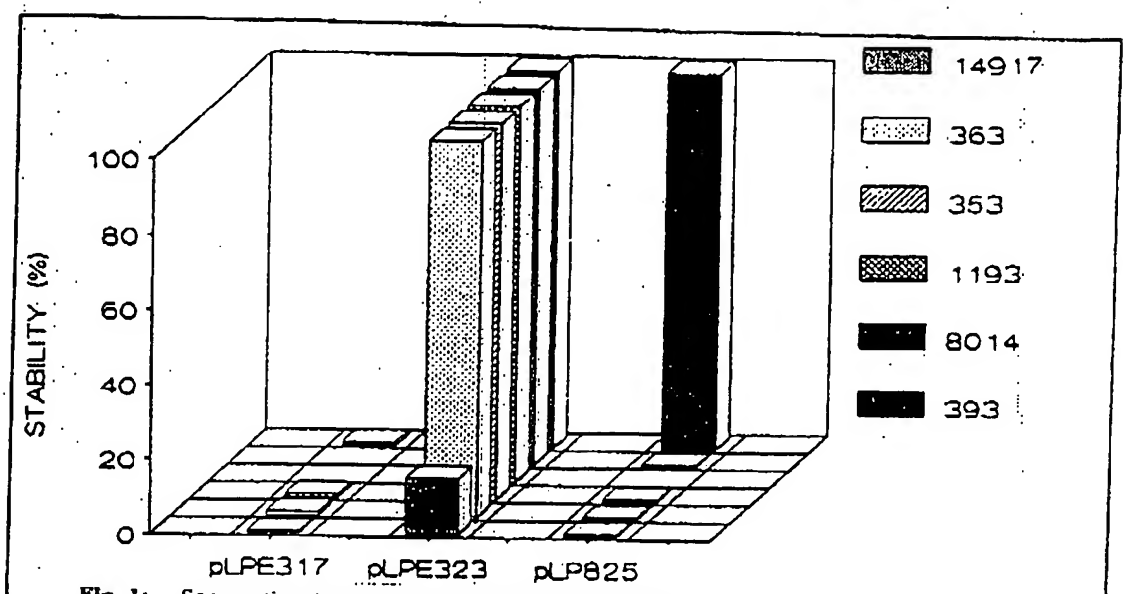

Fig. 1: Segregational stability of plasmid vectors pLPE317, pLPE323 and pLP825 (Posno et al, 1991b) in *L. plantarum* ATCC 14917, *L. pentosus* MD363, *L. pentosus* MD353, *L. plantarum* NCDO 1193, *L. plantarum* ATCC 8014 and *L. casei* ATCC 393. Stability is expressed as the percentage of resistance cells after 100 generations of cultivation in antibiotic-free medium.

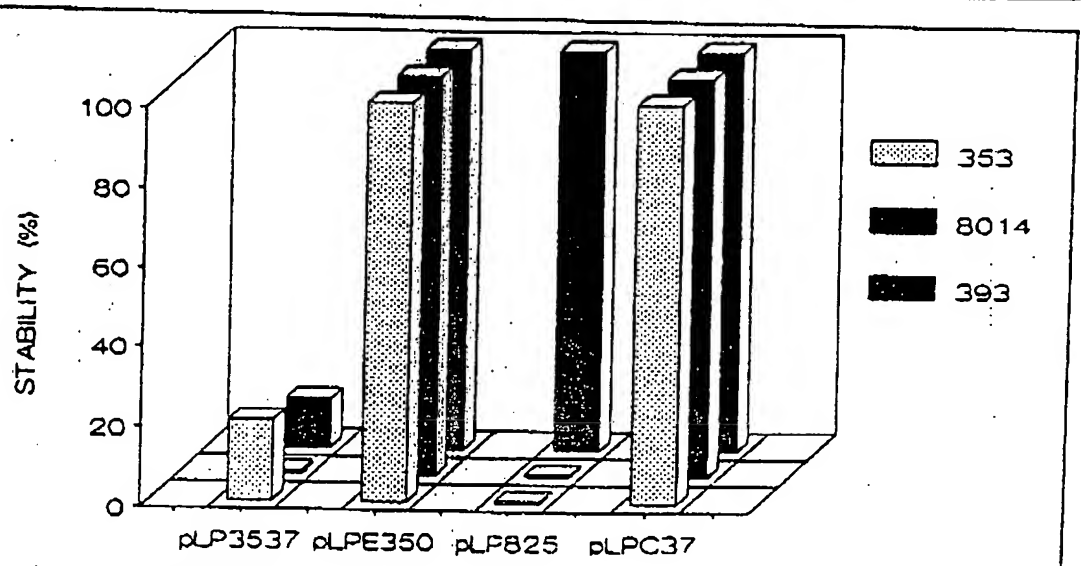

Fig. 2: Segregational stability of plasmid vectors with (pLP3537, pLP825) and without (pLPE350, pLPC37) *E. coli* sequences (Leer et al, 1992) in *L. pentosus* MD353, *L. plantarum* ATCC 8014 and *L. casei* ATCC 393. Stability is expressed as the percentage of resistance cells after 100 generations of cultivation in antibiotic-free medium.

selective conditions, harbours the same replicon as pLPE323 but differs from it by the presence of *E.coli* vector sequences. The difference in stability between the two plasmids can be fully accounted for by *E.coli* sequences. After removal of ≈95% of the *E.coli* sequences from pLP3537, the resulting vector, pLPE350, had become segregationally completely stable (Fig.2). A similar result has been obtained for two vectors based on a replicon from *L.plantarum*, pLP825 (with *E.*

coli sequences) and pLPC37 (without *E. coli* sequences), respectively (Fig.2). Segregational instability increases as the size of the inserted DNA fragment increases (Leer et al 1992), a phenomenon also observed in *B.subtilis* for plasmids derived from pUB110 (Bron et al 1990).

CHROMOSOMAL INTEGRATION

In cases where plasmid-borne genes are structurally or segregationally unstable, integration of cloned genes into the chromosome might offer a useful alternative to stabilize the gene (Chopin et al 1989; Leenhouts et al 1991). Scheirlinck et al (1989) have shown that the genes encoding endoglucanase from *Clostridium thermocellum* and α-amylase from *B.stearothermophilus* can be stably maintained and expressed in *L.plantarum* under non-selective conditions, after integration at an unknown site of the chromosome. Similarly, Leer and co-workers have demonstrated that replacement of the wild-type allele of the *L.plantarum* gene encoding bile-acid hydrolase by a mutant allele, by means of a gene-replacement experiment, results in a mutant phenotype which can be stably maintained under non-selective conditions for more than 100 generations (Leer et al 1993). To obtain transformants with an insertion into the chromosome, a high efficiency of transformation with replicating vectors is required, as the efficiency of transformation is three to four orders of magnitude lower than with replicating vectors. Alternatively, vectors may be used carrying a thermo-sensitive replicon. The broad-host-range plasmids pGhost (Maguin et al 1992) and pE194ts (Gryczan et al 1982) carrying a thermo-sensitive replicon derived from pGK12 and pE194, respectively, have been used for insertion of DNA fragments into the chromosome of *Bacillus* and *Lactococcus*. Since these vectors are also able to replicate in lactobacilli, they might be used for gene-tagging in these organisms as well.

CONSTRUCTION OF ANTIGEN-PRODUCING *LACTOBACILLUS* STRAINS: THE TOOLS

The application of *Lactobacillus* as an oral vaccine is, obviously, determined by a complex of factors, one of which is the capacity of a selected *Lactobacillus* strain to efficiently produce the antigen of choice. To this end, knowledge about sequences involved in (regulation of) gene expression in *Lactobacillus* is indispensable. Moreover, since it is unknown how an orally administered *Lactobacillus* strain must present the antigen to the immune system to generate an optimal immune response, knowledge about sequences which are needed to target the antigen to different locations of the cell (i.e intracellular, extracellular or surface-associated production of the antigen) also is of crucial importance.

A limited number of reports have been published describing the expression in *Lactobacillus* of genes from other bacterial sources under the control of their own promoter (Mercenier et al 1993; see also Table 4). Starting point of our strategy for the constructing of antigen-producing *Lactobacillus* strains, however, has been the use of homologous expression signals instead of heterologous ones.

During the past few years rapid progress has been made in the isolation and characterization of *Lactobacillus* genes. Consequently, knowledge about gene expression in *Lactobacillus* is beginning to rapidly emerge. Regulatory sequences have been identified, which have been shown to be very useful to achieve intracellular expression of heterologous genes in

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Table 4 HETEROLOGOUS GENE EXPRESSION IN *LACTOBACILLUS*

Protein	Origin	Species	Promoter
bile salt hydrolase	*L. plantarum*	*L. plantarum*	*cbh*
α-amylase	*L. amylovorus*	*L. plantarum / L. casei*	*amyA*
xylose isomerase/kinase	*L. pentosus*	*L. casei*	*xylA*
lipase	*S. hyicus*	*L. curvatus*	lipase
cellulase	*C. thermocellum*	*L. plantarum*	cellulase
xylanase	*C. thermocellum*	*L. plantarum*	xylanase
α-amylase	*B. amyloliquefaciens*	*L. plantarum*	amylase
α-amylase	*B. stearothermophilus*	*L. plantarum*	amylase
α-amylase	*B. thermosulfurogenes*	*L. plantarum*	amylase
glucanase	*B. amyloliquefaciens*	*L. helveticus*	glucanase
inulinase	*B. subtilis*	*L. plantarum*	*Ptac*
proteinase	*L. cremoris*	*L. casei*	proteinase
β-galactosidase	*E. coli*	*L. casei / L. plantarum*	*xylR, cbh*
β-glucuronidase	*E. coli*	*L. casei / L. plantarum*	*lacA, cbh*
FMDV-β-gal	FMDV / *E. coli*	*L. casei*	*xylR*
VP7-β-gal	Rotavirus / *E. coli*	*L. plantarum*	*cbh*
FMDV-α-amylase	FMDV / *L. amylovorus*	*L. casei*	*amyA*

Lactobacillus. Substantial research efforts are currently be carried out to identify, characterize and apply regulatory sequences for secretion of heterologous proteins into the medium or to the cell-surface. In the paragraphs below we will discuss the state-of-the-art with regard to regulatory signals involved in gene expression in *Lactobacillus*, development of expression vectors for *Lactobacillus* and different strategies exploiting the potential of *Lactobacillus* strains to express heterologous genes, in particular genes coding for antigens.

GENE EXPRESSION IN *LACTOBACILLUS*: REGULATORY SIGNALS

To date more than sixty-five *Lactobacillus* genes have been cloned and their nucleotide sequence determined. The genes are derived from more than ten species. Because of the heterogeneity of these species (reflected by considerable differences in overall G+C content among different lactobacilli), care should be taken in drawing general conclusions about the structure of *Lactobacillus* expression signals, such as promoters, terminators and ribosome binding sites, etc. On the other hand, it has been shown in many cases that *Lactobacillus* genes can be functionally transferred to other *Lactobacillus* species and even *E. coli*, indicating that transcription and translation signals of such genes are sufficiently similar to be recognized in taxonomically quite distinct microorganisms (e.g. Lerch et al 1989; Posno et al 1991a).

TRANSCRIPTION

We have followed two different approaches to isolate suitable *Lactobacillus* promoters. In the first approach, DNA fragments exhibiting promoter activity were been obtained by shotgun cloning of *Lactobacillus* chromosomal DNA fragments upstream of a promoterless chloramphenicol resistance gene (promoter-probe vector). This strategy, which is fast and relatively simple, has the additional advantage that it selects for strong and most likely constitutive promoters. A number of promoter activity containing *Lactobacillus* chromosomal DNA fragments have been characterized into more detail with respect to relative promoter strength (analysis of chloramphenicol acetyl transferase activity) and promoter structure (primer extension analysis). The results showed that the structures of the promoters are quite similar, although significant differences in relative promoter strength were observed. An important disadvantage of this approach, however, is that nothing is known of possible regulation mechanisms of the promoter in concern. Therefore, the majority of our research efforts have been concentrated on the second approach which deals with the analysis of upstream regions of well-characterized *Lactobacillus* genes, an example of which is described in the next paragraph.

We have cloned five genes involved in xylose catabolism in *L. pentosus* and have determined the transcriptional organization of the genes (Lokman et al 1991, Lokman et al, in preparation). Induction (by xylose) of the *xylA/B* operon, which encodes xylose isomerase (A) and xylulose kinase (B), takes place at the level of transcription and is negatively controlled by a repressor, the product of *xylR*. The promoters of the *xylR* gene and *xylA/B* operon, were identified by mapping of the 5' ends of the messengers by primer extension analysis (Lokman et al, in preparation) and compared with promoter sequences of other *Lactobacillus* genes (Fig.3).

Transcription startsites

Fig. 3: Consensus sequence of transcription startsites of *Lactobacillus* genes. Frequence of occurence of nucleotides: large bold capitals: > 75%; capitals: 50 - 75%; small capitals: most frequently encountered nucleotide.

To date, transcription start sequences have been identified for eleven genes originating from seven different species. Compilation of *Lactobacillus* promoters reveals the presence of sequence elements which are similar or even identical to the consensus sequence ('-35': TTGACA -17bp- '-10': TATAAT) of Gram-positive and Gram-negative promoters (Moran et al 1982; Graves & Rabinowitz 1986; Fig.3). The length of the untranslated region of most transcripts is relatively small (<100 nt), comparable to that found in *E. coli* and *B. subtilis*. The region 5' to the -35 element is rich in A residues (Fig.3), as has been observed for *Bacillus* promoters that are recognized by σ-70 factors (Moran et al 1982). The spacer region between the conserved hexanucleotides is rich in A and

T residues (Fig.3). A similar consensus can be derived from the nucleotide sequence of the upstream regions of *Lactobacillus* genes from which the promoter has not been mapped yet. It should be noted, however, that in these cases it is uncertain whether these sequences represent functional promoter elements.

At the 3' end of most *Lactobacillus* genes a sequence element is present showing features that are similar to those of rho-independent-like terminators. From our own work it has appeared that insertion of the presumed terminator sequence of the *xylA/B* operon of *L. pentosus* downstream of a marker gene results in a transcript that is terminated at the site of the terminator, reinforcing the conclusion that such elements indeed represent functional terminators (Lokman et al, in preparation).

TRANSLATION AND CODON USAGE

The nucleotide sequences around the translation-initiation sites of approximately seventy *Lactobacillus* genes are known. Comparison of these sequences reveals the presence of a highly conserved sequence (AGGAGG), the Shine-Dalgarno (S-D) motif, at a distance of 6-10 nucleotides from the initiation codon.

The S-D motif of different *Lactobacillus* species is conserved and similar to that of most

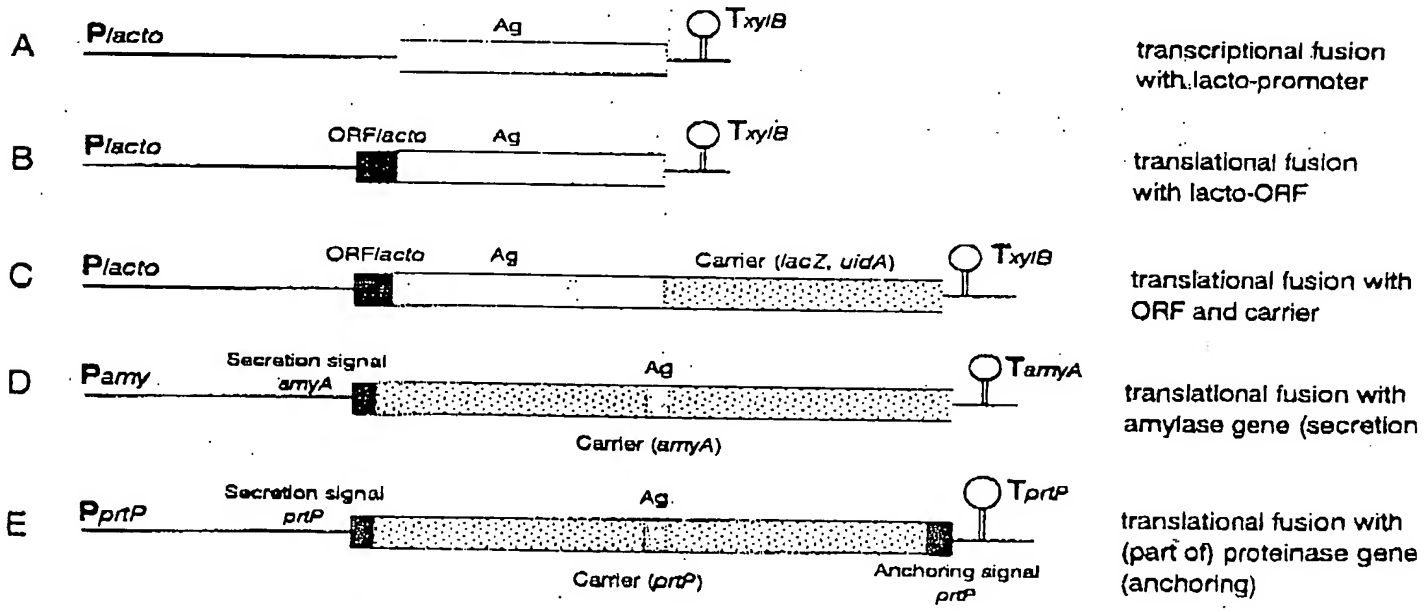

Fig. 4: Structure of expression cassettes for antigen production in *Lactobacillus*. Ag: gene coding for antigen of interest; *Placto*: *Lactobacillus* promoter; *PamyA*, *PprtP*: promoters of *L. amylovorus* α-amylase gene and *L. casei* proteinase gene, respectively, *TxylB*, *TamyA*, *TprtP*: transcription terminators of *L. pentosus xylB* gene, *L. amylovorus* α-amylase gene and *L. casei* proteinase gene, respectively; ORF*lacto*: 10-20 codons of *Lactobacillus* open reading frame; *lacZ* (*E. coli*), *uidA* (*E. coli*), *amyA* (*L. amylovorus*), *prtP* (*L. casei*): genes encoding carrier proteins β-galactosidase, β-glucuronidase, α-amylase and (part of) proteinase, respectively.

other genes of Gram-negative and Gram-positive origin. In contrast, codon usage of *Lactobacillus* genes shows a clear bias for specific codons. For example, codon usage in two-codon sets is clearly different for two different *Lactobacillus* species: *L. bulgaricus* and *L. plantarum*. Moreover, efficiently expressed *Lactobacillus* genes generally show a marked preference for C over U, and G over A in the third position of the codon in two-codon sets. Finally, evidence is accumulating that codon usage in the extreme 5' end of *Lactobacillus* genes may not be random either. Anticipating on the last mentioned observation, in most of our expression vectors for antigen production, the "*Lactobacillus* translation-initiation region" is maintained by making translational fusions of the antigen of interest with the first 10-20 codons of a *Lactobacillus* open reading frame (see Fig.4), instead of placing the antigen under direct control of a *Lactobacillus* promoter (transcriptional fusion).

PROTEIN SECRETION AND MEMBRANE-ANCHORING

Knowledge about *Lactobacillus* sequences which could mediate targeting of the antigen to the medium or, preferably, to the cell membrane or cell wall, is beginning to evolve.

Several *Lactobacillus* species are known to produce extracellular enzymes like α-amylase, inulinase and proteinase (Burgess-Casler & Imam 1991; Szilágyi pers. comm.; Holck & Naes 1992). In order to gain more information on protein routing and secretion in lactobacilli, we have isolated the α-amylase gene of one of the few *Lactobacillus* species that is capable to grow on starch: *L. amylovorus*. The α-amylase from *L. amylovorus* contains a signal sequence that is typical for secreted proteins. The signal sequence of α-amylase from *L. amylovorus* is 49 amino acids long, somewhat larger than the *B. subtilis* enzyme (Jore et al, in preparation). Similarly, a proteinase from *L. paracasei* (Holck & Naes 1992) and two surface-layer proteins (Vidgrén et al, Boot et al 1993) contain signal sequences that are typical for exported proteins. In view of these observations it is of no surprise that signal sequences from enzymes of Gram-positive origin have been shown to function in *Lactobacillus*. Genes coding for *B. amyloliquefaciens* α-amylase (Jones & Warner 1990), *C. thermocellum* cellulase and xylanase (Scheirlinck et al 1990) or *S. hyicus* lipase (Vogel et al 1990) are all expressed in *Lactobacillus* and their products secreted under control of their own regulatory sequences (see also Table 4).

The *prtP* gene of *L. paracasei* encodes a cell-associated proteinase (Holck & Naes 1992), which shares extensive similarity with the *Lactococcus lactis* subsp. *cremoris* Wg2 proteinase (Kok et al 1988). It has been shown that the C-terminal part of the proteinase is not required for proteinase activity but encodes a membrane anchor with homology to the membrane anchors of cell-associated proteins from distantly related species, like staphylococcal protein A and streptococcal protein M6 (Fischetti 1993). The *L. paracasei* proteinase gene has been expressed from its own regulatory signals (promoter, secretion signal, membrane anchor) in *L. plantarum* (Holck & Naes 1992). We are currently exploring the applicability of the membrane anchor of

L. paracasei as a signal to target foreign antigens to the cell-surface of *Lactobacillus* (Fig.4).

EXPRESSION VECTORS

A variety of expression vectors is now available for *Lactobacillus*, that are based on plasmids and regulatory sequences derived form *Lactobacillus* (Fig.4). A typical example is the vector pLPCR2, which is based on the regulatory sequences of the xylose genes of *L. pentosus*. Briefly, the promoter of the *xylR* gene and the terminator of the *xylB* gene (separated by a multiple cloning site) were inserted in the *E. coli - Lactobacillus* shuttle-vector pLP3537 (Posno et al. 1991b). The resulting vector was designated pLPCR2. The *xylR* promoter in pLPCR2 has been successfully used to express heterologous genes in different *Lactobacillus* strains. Similarly, we have successfully used the regulatory sequences of the *L. plantarum* conjugated bile acid hydrolase gene (*cbh*), the *L. casei* L-lactate dehydrogenase gene (*ldh*) and *L. amylovorus* α-amylase gene (*amyA*) for the construction of expression vectors for intracellular and extracellular production of antigens (Fig.4).

HETEROLOGOUS GENE EXPRESSION: PRODUCTION OF (MODEL) ANTIGENS

A gene encoding protein p72 was inserted in the polylinker of pLPCR2 and subsequently introduced into *L. casei* by electroporation. Protein p72 consists of two copies of a foot-and-mouth-disease virus (FMDV) epitope (in tandem) which are fused with *E. coli* β-galactosidase (βgal) (Broekhuijsen et al 1986). Synthesis of p72 could be detected in cellfree extracts of transformed cells both with antibodies specific for the FMDV epitope and antibodies specific for βgal (Fig.5). The level of expression, however, was relatively low (about 0.1% of total cellular protein). The low level of expression may be one of the explanations why intraperitoneal immunization of mice with heat-killed cells of the transformed *L.casei* strain did not elicit a significant humoral immune response against the fusion protein.

In an attempt to try to increase the expression level, a new vector was constructed based on a promoter that was assumed to be stronger than that of *xylR*. Using the regulatory sequences of the *cbh* gene of *L. plantarum*, we had previously demonstrated that a 10-fold overproduction of a homologous protein in *L. plantarum* could be achieved (Christiaens at al. 1992). Therefore, this promoter was chosen for further expression studies. First, the *E. coli lacZ* gene was placed under control of the *cbh* promoter and the sequence encoding the N-terminus of Cbh (translational fusion). *L. plantarum* transformants containing the resulting vector produced βgal to about 1-2% of total cellular protein, indicating that the *cbh* promoter is a suitable promoter for heterologous gene expression in *L. plantarum*. In a pilot study, intraperitoneal immunisation of mice with the *L. plantarum* strains resulted in the generation of a significant primary IgM immune response against βgal and, after a booster dose, a systemic IgG response was found. These results show that the βgal protein is produced by *L. plantarum* to levels that are sufficient

to trigger antibody response.

While the *cbh* promoter is a suitable promoter for the production of a model antigen in *L. plantarum*, it is not in another host, *L. casei*. Introduction of the expression vector from *L. plantarum* into *L. casei* only yielded transformants that did not produce βgal. Apparently, the activity of some *Lactobacillus* promoters is strain-specific. Moreover, when rotavirus capsid protein VP7 was fused to the N-terminus of βgal, it appeared that the level of expression in *L. plantarum* was drastically reduced compared to βgal alone (about 0.2% of total cellular protein). Clearly, the level of production is influenced by the nature of the gene product that is produced. Strong evidence has been obtained that in certain cases constitutive expression of a heterologous gene may even be lethal to the *Lactobacillus* host. For example, we have found that a gene coding for hepatitis delta virus antigen (HDVAg) could only be introduced into *L. casei* in the case that this gene was not preceded by functional regulatory sequences. However, transformation of *L. casei* with the same expression cassette under control of a functional promoter and ribosome binding site yielded only a few transformants which could not be further cultivated. To be able to produce proteins like HDVAg, regulatable expression systems for intracellular and extracellular production could offer an alternative. An attractive candidate is the promoter of the α-amylase gene of *L. amylovorus*, whose activity can be induced by for instance maltose and almost completely repressed by for instance glucose. Recently, we have succeeded in the controlled extracellular production of a fusion protein consisting of α-amylase of *L. amylovorus* and a foot-and-mouth disease epitope.

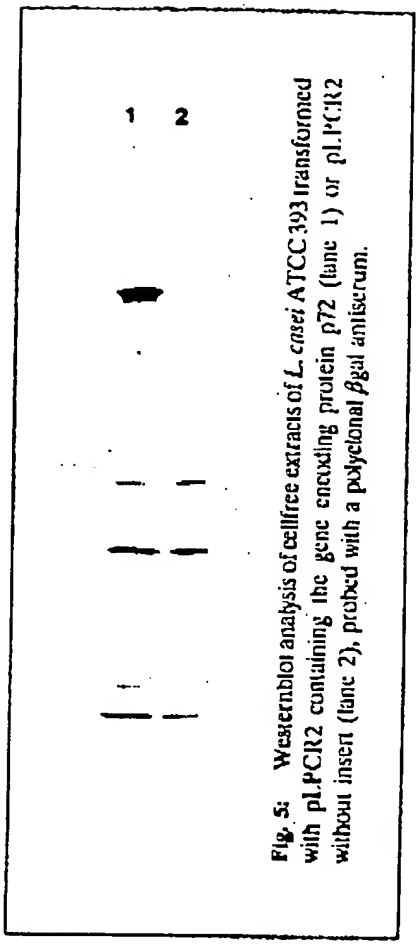

Fig. 5: Westernblot analysis of cellfree extracts of *L. casei* ATCC 393 transformed with pLPCR2 containing the gene encoding protein p72 (lane 1) or pLPCR2 without insert (lane 2), probed with a polyclonal βgal antiserum.

ADJUVANTICITY OF *LACTOBACILLUS*

For *Lactobacillus* various so called probiotic properties have been described after oral administration, including the capacity to stimulate the immune system in an aspecific manner i.e adjuvanticity. The role of *Lactobacillus* as an adjuvant may originate in aspecific stimulation or in multiple determinant antigen presentation. Main goal of the present experiments was the selection of a particular *Lactobacillus* strain with a profile of properties as required for candidate vaccine carriers. As model antigens we used trinitrophenylated chicken gamma globulin (TNP-CGG) or synthetic peptides.

For intraperitoneal immunization the mixture of antigen and *Lactobacillus* with supposed adjuvant activity were administered in PBS, antigens were also administered following the same route using Specol, a water in oil adjuvant, as a positive control (Boersma et al., 1992). Two immunizations with a four week interval were performed. Adjuvant activity of various strain was determined in a humoral immune response analysis (by ELISA) to CGG-TNP after intraperitoneal administration. Figure 6 shows that after the first immunization (day 14) strain dependent adjuvanticity was observed. *L. casei* were almost as potent in supporting the IgG immune response to CGG-TNP as was the water in oil adjuvant specol. Other strains like *plantarum* and *acidophilus* showed an intermediate adjuvanticity. Results of serum evaluation on a coating of OVA-TNP gave similar results for TNP specific responses. Secondary responses to the antigen again administered with the same strain and dose of *Lactobacillus* did not reveal much differences in adjuvanticity of the strains used and the results did not differ greatly between the control adjuvant Specol and the *Lactobacillus* as an adjuvant (not shown).

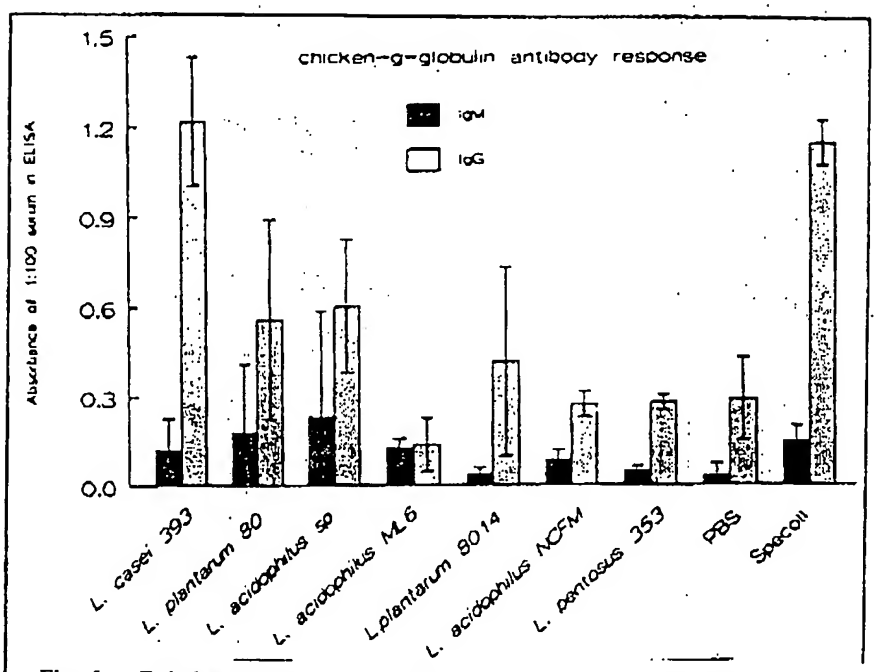

Fig. 6: Relative adjuvant activity of *Lactobacillus* strains. Mice were immunized with an admixture of *Lactobacillus* (10^9) and 50 μg of antigen in PBS. ELISA responses of serum samples diluted 1:100 from day 14 after the first immunization indicate relative IgM and IgG responses to a coating of CGG.

The cell mediated immune response was determined as a DTH reaction to a subcutaneous challenge with the protein CGG-TNP. Figure 7 (see also table 5) shows the results determined at 48 and 72 h after challenge. *L. casei* and *L. plantarum* gave the best results in this type of response, showing that Lactobacilli can be potent adjuvants comparable to water in oil emulsions.

CARRIER FUNCTION (PROVIDER OF T-CELL-EPITOPE) OF *LACTOBACILLUS*

Recently we have shown that *Lactobacillus* strains can effectively be used as carriers for antigens (Gerritse et al. 1991a,b; Claassen et al. 1993a-c). We were able to demonstrate humoral immune responses after oral immunisation, followed by intraperitoneal boosting, with heat

inactivated *Lactobacillus*-antigen conjugates. In those studies the hapten TNP (trinitrophenyl) was chosen as a model antigen because it could be easily coupled to the surface of *Lactobacillus* via free amino residues on the bacterium. Furthermore, TNP by itself will not (by definition as a hapten) induce an immune response, any response against TNP will result from a T-cell-epitope provided by proteins from *Lactobacillus*. By making use of TNP enzyme conjugates we could also study the local immune response by detecting specific anti-TNP antibody forming B-cells *in situ* in the GALT and mesenterical lymph nodes (Claassen et al. 1991). In those studies, primary responses were mainly of the IgM and IgG isotype, almost no IgA was detected. IgM peaked at around day 7 and was almost back to baseline levels after three weeks whereas IgG gradually increased with time. Upon oral boosting all isotypes were detected and a clear IgG memory response could be demonstrated. This immune response was as high as those obtained

LACTOBACILLUS STRAINS USED IN ADJUVANT AND CARRIER EXPERIMENTS

No	Strain	Origin	No.	Strain	Origin
1.	L. acidophilus IV, ML25	mouse	2.	L. fermentum a, R37	pig
3.	L. casei shirota	unk.	4.	L. plantarum	ATCC8014
5.	L. fermentum I, ML1	mouse	6.	L. delbrueckii b, R81	pig
7.	L. fermentum b, R49	pig	8.	L. brevis a, R3	pig
9.	L. nd., III	mouse	10.	L. acidophilus	NCFM

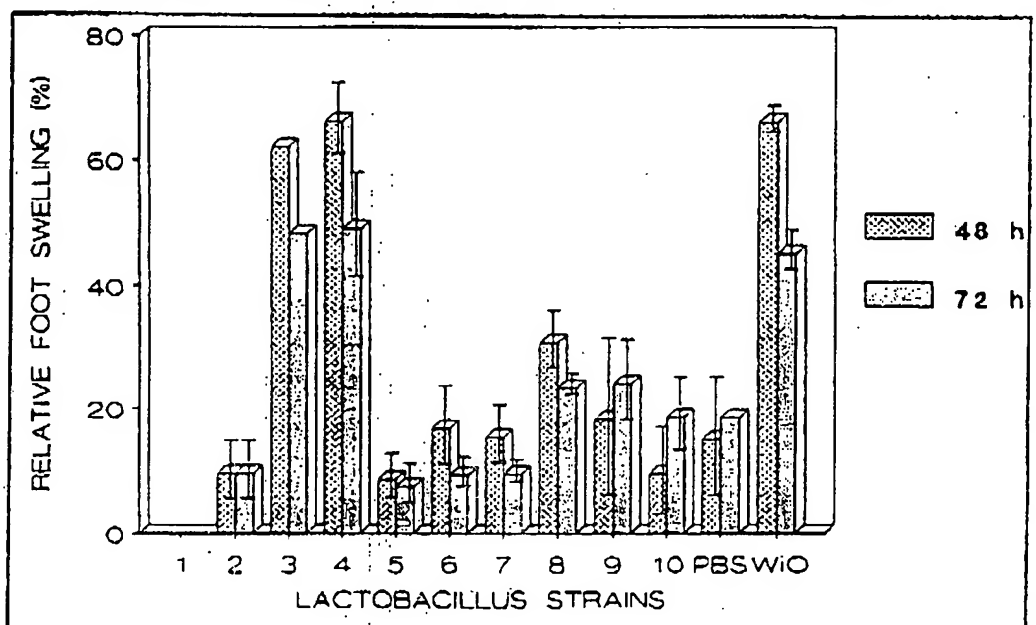

Fig. 7: DTH responses to a subcutaneous challenge of previously i.p. immunized mice. The haptenated protein CGG-TNP was applied in the hind foot in 50 μl in PBS only. Responses were determied as relative foot swelling (Vissinga et al., 1989).

by injection of the conjugate through routes that are considered to be more efficient (intravenous, intraperitoneal). However, immunisation by i.v. or i.p. routes never resulted in IgA type responses, as expected. Furthermore, virtually no immune response, and certainly no IgA, against the microorganism itself was observed after repeated oral immunisation. Analysis of the local response by detection of TNP specific AFC showed that most of the antibodies were produced in the spleen and mesenterical lymph nodes, no cells were found in the Peyers patches and only very few in the lamina propria of the gut. Immune complexes consisting of TNP-*Lactobacillus* and antibodies, directed against TNP and against the bacterium, were found exclusively in the mesenterical lymph nodes. Immune complexes were located in the B-cell-follicles bound to the follicular dendritic cells. Complex deposition started at day 5 (following IgG formation) and complexes persisted for at least 4 weeks (later time points not tested) no complement mediated immediate trapping was observed (Gerritse et al. 1991b; Claassen et al. 1993a).

To further evaluate the observed effect of particular *Lactobacillus* strains as immunogenic carriers and/or adjuvants, *Lactobacillus* of all strains evaluated (table 5) were trinitrophenylated and the response to the hapten was determined in ELISA with OVA-TNP as a coating. As figure 8 shows, also in this case *L. plantarum* was an effective carrier for surface exposed antigen (TNP).

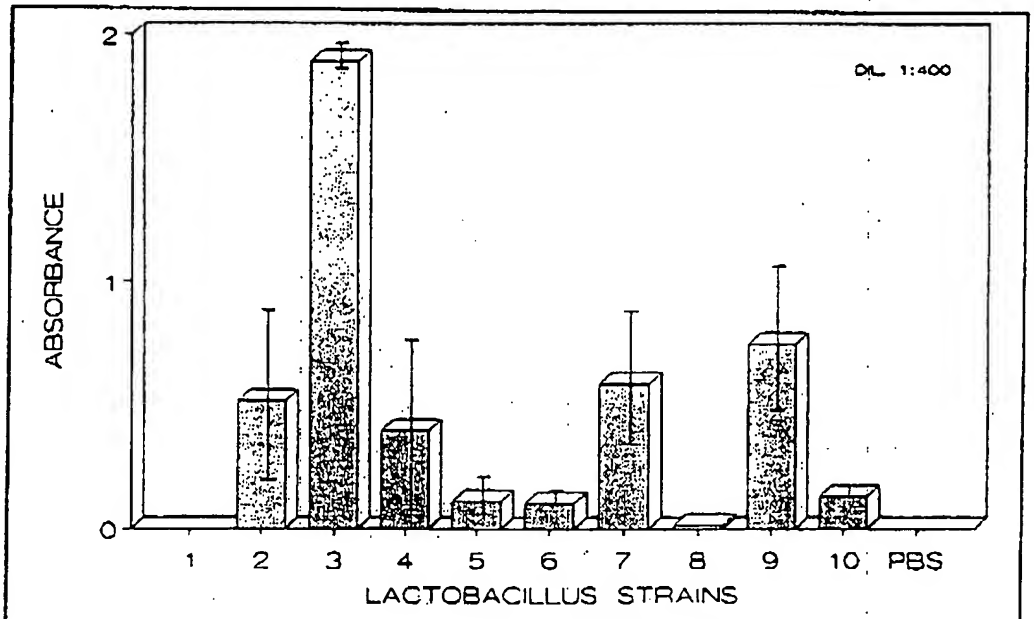

Fig. 8: Typical responses in serum (dilution shown 1:400) of mice twice immunized with *Lactobacillus*-TNP conjugates (10^9). Relative IgG responses to a coating of OVA-TNP are shown. The responses shown were determined day 8 after the second immunization.

To investigate whether the immune responses, observed with Lactobacilli-peptide-constructs in pilot studies, were aided by intrinsic adjuvant activity or by carrier function (as for TNP) of the bacteriae, we used a synthetic peptide comprising both a T- and B-cell epitope which (when adjuvated with a water-in-oil emulsion,

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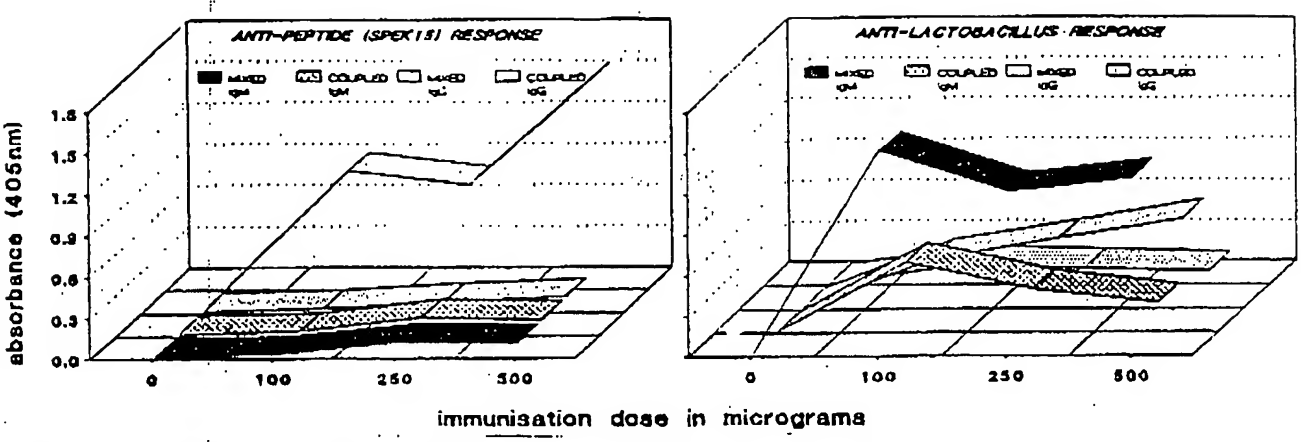

Fig. 9: Anti-peptide and -lacto responses after immunization. SP 15 a was administrated together with Lactobacilli (10^9 L + SP15a resp. 100, 250 or 500 μg peptide) or coupled to the bacteria using a chemical coupling which uses the -NH2 groups of *Lactobacillus* surface proteins (L-SP15a). The results expressed here were obtained with the synthetic peptide as a coating. As for the protein experiments, IgM responses are relatively low. Only with coupled peptides IgG responses could be evoked. Responses shown were obtained day 7 after the second immunization

Boersma et al. 1992) could induce antibodies crossreactive with the peptide and the native protein it was derived from (Zegers et al. 1992). As figure 9 shows, admixing of SP does not result in significant IgG or IgM responses, only conjugation of the SP to the Lactobacilli results in acceptable IgG responses. This response is not dose dependent although variation in results (expressed as s.d.) is clearly less with the highest amount. The significant decrease of anti-Lactobacilli (both IgM and IgG) titers observed when SP are covalently linked (instead of admixed, panel B) serves as an internal control on the coupling efficiency of the SP and also indicates that non-Lactobacillus structures on the surface mask endogenous determinants.

In conclusion: Lactobacillus strains (mouse and human derived) showed adjuvant properties to a level as observed for water in oil (WIO) emulsions, Some strains showed no adjuvanticity at all but no direct relationship between host origin and adjuvanticity could be determined. Adjuvanticity was found with CGG-TNP as the antigen but not with low molecular weight antigens: synthetic peptides with one B cell and one T cell epitope. The same peptides coupled to *Lactobacillus* were highly immunogenic proving that the bacterium can effectively provide T-cell help for small epitopes.

VEHICLE FUNCTION OF *LACTOBACILLUS*

Antigen Uptake And Routing Into The Immune System

Normally there are four possible ways of antigen uptake by the gut. 1) nonspecific uptake of antigen between epithelial cells in instances where the gut is damaged 2) nonspecific uptake via epithelial cells 3) receptor-ligand mediated uptake and 4) specialised uptake via M-cells in the Peyers patches (cf. Van der Heijden 1990). As shown before no uptake or association of

Lactobacilli with structures other than M-cells in the gut was observed in numerous experiments we performed. Lactobacilli were rapidly taken up by M-cells from the Peyers patches (Claassen et al. 1993b) after 6-12 hrs they were observed in the dome area and no label was seen after 48 hours. From that time Lactobacilli could be demonstrated in the mesenterical lymph nodes (in the same area as the medullary macrophages). With a brush border adhesion assay we proved that the Lactobacilli used (*L. acidophilus* NCK'89) did not adhere to the gut.

Colonisation Of The Gut

It is still not clear whether persistence of a live microorganism is an advantage for oral immunisation or a disadvantage. The latter might be so because of the fact that the antigenic determinant will be exposed for prolonged periods of time in the gut, possibly leading to the induction of tolerance. In view of the good results we obtained with Lactobacilli we decided to investigate whether the strain we used for the TNP experiments colonized the gut or not. Live *L. acidophilus* NCK'89 (made streptomycin resistant) were given orally at a dose of $5 \cdot 10^7$. Gut sections of duodenum, upper-ileum, lower-ileum, appendix and colon were removed (after 1 and 5 days) and gut content was suspended and plated on rogosa plates with and without streptomycin. colony forming units were determinated after incubation. After one day over 1000 CFU of Lactobacilli were found in all gut segments. Experimentally administered Lactobacilli (streptomycin resisitant) were found almost exclusively in appendix and colon (500 and 200 CFU resp.). After five days, streptomycin sensitive (as day 1) but no resistant CFU could be detected from any of the gut segments.

IMMUNE RESPONSES AGAINST LIVE TRANSFORMED LACTOBACILLI

In view of the good results obtained in the above studies, *L. plantarum 80* was transformed to overexpress β-galactosidase and given orally to mice three times (day 0, 1, 2) and once after a 4 week interval. 10^6 - 10^{10} bacteriae were given. As a control 10^{10} *Lacto's* were given intraperitoneally. *L.plantarum 80* which were non-transformed were used for comparison. ELISA data for the anti-β-galactosidase activity are shown, the bars figures represent the responses of individual mice. From figure 10 can be concluded, that in the intestinal lavages no significant Ig (total) responses to β-galactosidase was obtained in ELISA after oral administration of the bacteriae when the results were compared to i.p. administration of the same bacteriae. No difference with administration of bicarbonate only was observed. IgA responses were found in intestinal lavages after administration of the highest dose of *Lactobacillus* only (fig 11). Though the responses remain relatively low the results at the presently obtained, realtively low, level of overexpression of β-galactosidase are encouraging. Serum antibody responses (figure 12) after the first oral immunizations are low as compared to the i.p. administration of the same dose of antigen. In the booster reaction however almost no significant levels of antibody responses were

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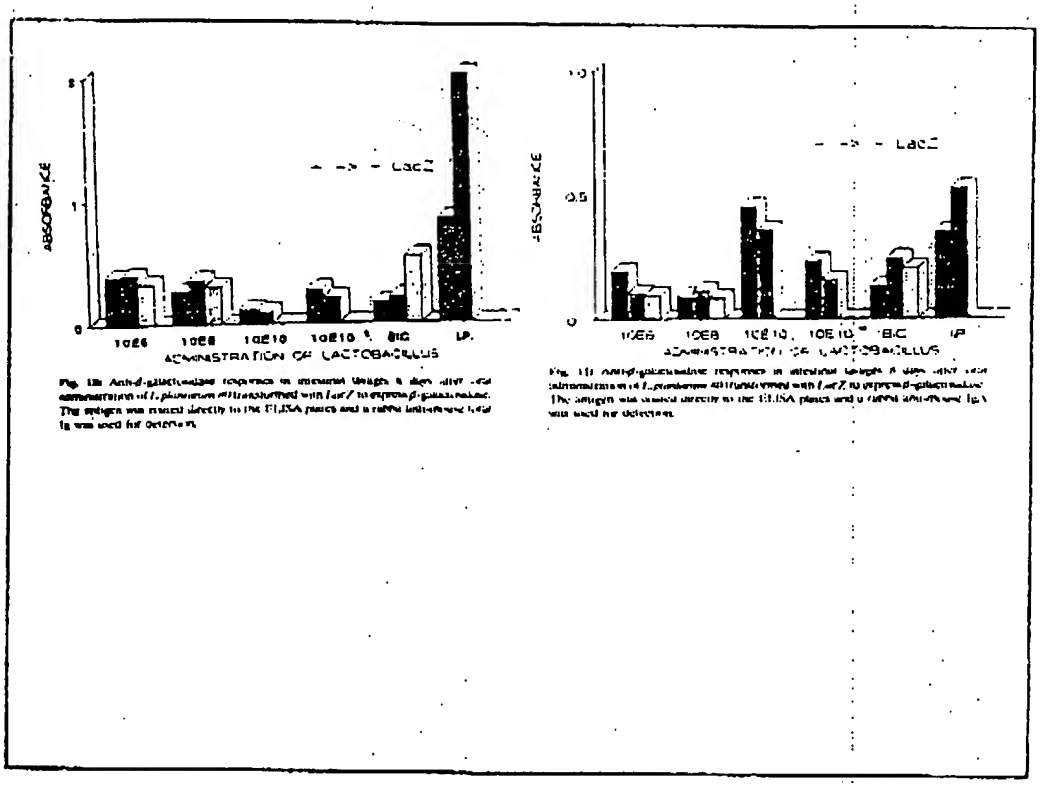
determined. This might be an indication for the induction of a mild immune response (prime day 7, 14 and boost day 4), which upon a second application of the antigen turns into a form of tolerance. This phenomenon was not found with surface exposed antigens/haptens suggeting that secreted or intracytoplamic antigens are not a proper form for expression in Lactobacilli to be used as vaccines.



CONCLUSIONS

Both from the results obtained with the hapten TNP as well as from the SP it is clear that *Lactobacillus* can offer carrier function (i.e. T cell epitope) to structures covalently linked to its surface. Even more important than the excellent antibody responses we found was the fact that systemic memory for IgA and IgG for the antigen could be induced with *Lactobacillus*-conjugates when given orally. Precise evaluation showed, that the response was mainly directed to the T determinant of the peptide which was most exposed. (coupling site was on the B cell part of the peptide). It should be emphasised that the SP used contained a functional T and B epitope and was immunogenic by itself when administered i.p. with FCA or Specol. Consequently the lack of response in the group where SP was admixed with *Lactobacillus* clearly indicates the lack of adjuvant effect under the present experimental conditions (e.g. strain, route, dose, antigen).

Contrary to the SP constructs, upon administration of TNP-*Lactobacillus* no anti-*Lactobacillus* response was observed. This was due either to epitope masking because of heavy labeling with TNP or to the route used (i.e. oral for TNP and i.p. for SP). It is also noteworthy to see that the IgM titers against free *Lactobacillus* were high also after boost, this might be

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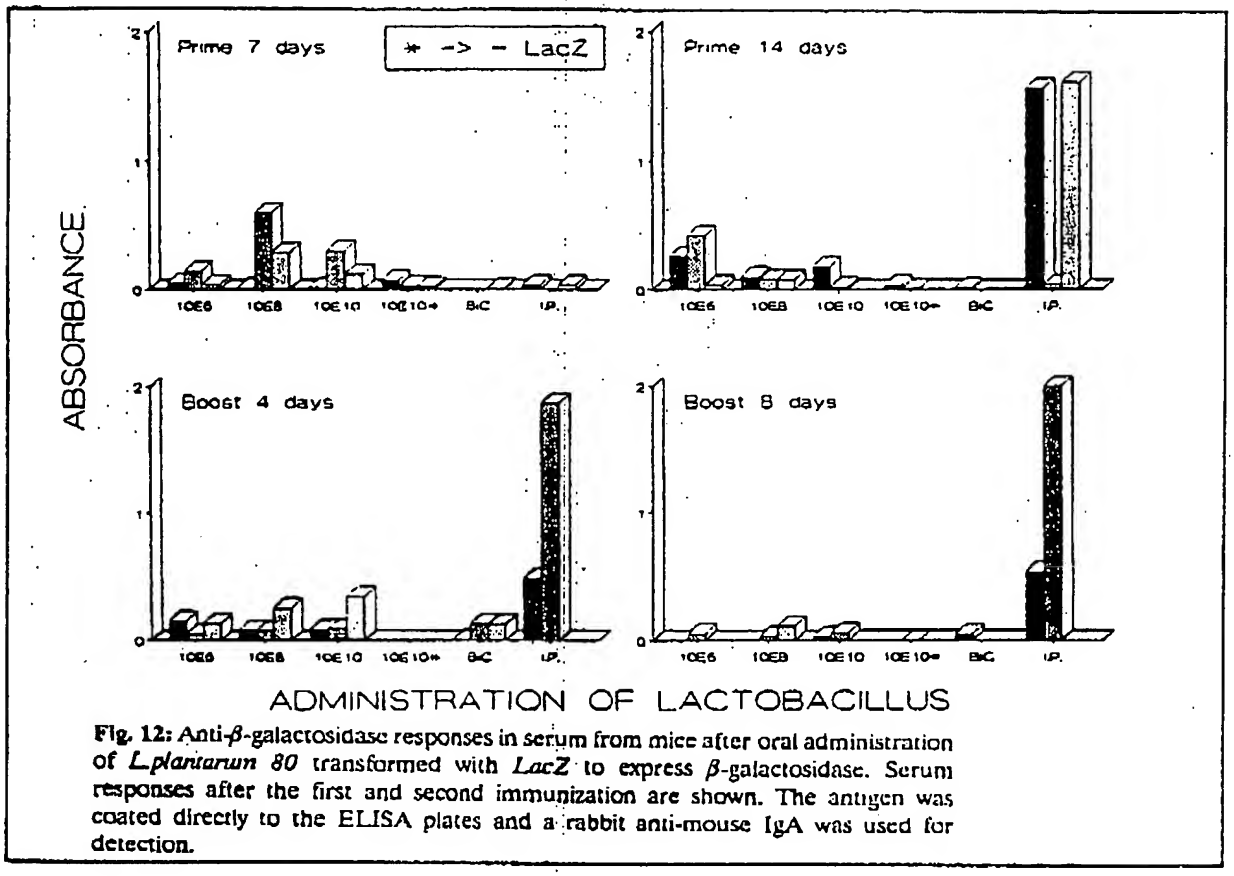

Fig. 12: Anti-β-galactosidase responses in serum from mice after oral administration of *L.plantarum 80* transformed with *LacZ* to express β-galactosidase. Serum responses after the first and second immunization are shown. The antigen was coated directly to the ELISA plates and a rabbit anti-mouse IgA was used for detection.

explained as being the result of a T independent response induced by glycosylated groups of surfaceproteins. Surprisingly this observed anti-*Lactobacillus* response is not present in memory IgG and IgA type responses (after boost IgM only).

The level of expression of antigens in transformed *Lactobacillus* has to be increased to initiate satisfactory responses along the mucosa. In addition we are in the process of development of cell wall associated antigens (through membrane anchors). The role of colonization ability of the *Lactobacillus* used as a vaccine carrier has not yet been investigated in great detail, recolonization of the recombinant strain may be necessary and colonization abilities might be lost after transformation.

The new data presented in this study clearly support the view that Lactobacilli can be efficiently used as a carrier for oral vaccination, more work on strain selection (adjuvanticity) and expression of surface proteins in relation to immune response and protection against selected pathogens is still needed.

ACKNOWLEDGEMENTS

The authors wish to thank: Antoon Van Den Bogaerdt, Jan Jore, Marijke Kottenhage, Rob Leer, Christien Lokman, Cees Lucas, René van Winsem and Netty Zegers for their invaluable contributions which made this work possible and for being such fine colleagues.

REFERENCES

Ahrné, S., Molin, G., & Axelsson, L. (1992). Transformation of *Lactobacillus reuteri* with electroporation studies on the erythromycin resistance plasmid pLUL631. *Curr. Microbiol.*, 24, 199-205.

Aukrust, T., & Nes, I. F. (1988). Transformation of *Lactobacillus plantarum* with the plasmid pTV1 by electroporation. *FEMS Microbiology Letters*, 52((1-2)), 127-132.

Boersma W.J.A., Bogaerts W.J.C., Bianchi A.T.J. and Claassen E. (1992) Adjuvant properties of stable water in oil emulsions: evaluation of the experience with specol. *Res.Immunol.* 143, 503-512.

Boersma W.J.A., Haaijman J.J. and Claassen E. (1993) Use of synthetic peptide determinants for the production of antibodies. In: *Immunohistochemistry 2nd Edition*. Editor. A. C. Cuello. John Wiley & sons, 1-77.

Bloom B, (1985) Vaccines for the Third World. *Nature*, 342, 115-120.

Boot, H., Kolen, K.P.A.M., van Noort, J.M., & Pouwels, P.H. (1993). S-layer protein of *Lactobacillus acidophilus* ATCC 4356: Purification, expression in *Escherichia coli*, and nucleotide sequence of the corresponding gene. *J. Bacteriol.*, in press.

Bouia, A., Bringel, F., Frey, L., Kammerer, B., Belarbi, A., Goyonvarch, A., & Hubert, J. C. (1989). Structural organization of pLP-1, a cryptic plasmid from *Lactobacillus plantarum* CCM1904. *Plasmid*. 22(3), 185-192.

Bringel, F., Frey, L., & Hubert, J. C. (1989). Characterization, cloning, curing, and distribution in lactic acid bacteria of pLP1, a plasmid from *Lactobacillus plantarum* CCM 1904 and its use in shuttle vector construction. *Plasmid*, 22(3), 193-202.

Broekhuijsen, M.P., Blom, T., Kottenhagen, M., Pouwels, P.H., Meloen, R.H., Barteling, S.J., & Enger-Valk, B.E. (1986). Synthesis of fusion proteins containing antigenic determinants of foot-and-mouth disease virus. *Vaccine*, 4, 119-124.

Bron, S., Luxen, E., & Swart, P. (1988). Instability of recombinant pUB110 plasmids in *Bacillus subtilis*, plasmid-encoded stability function and effects of DNA inserts. *Plasmid*, 19, 231-41.

Brown F. (1989) Vaccination. *Current Opinion in Immunology*, 1, 467-474.

Brückner, R. (1992). A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. *Gene*, 122, 187-192.

Burgess-Casler, A., & Imam, S. (1991). Partial purification and comparative characterization of α-amylase secreted by *Lactobacillus amylovorus*. *Current Microbiol.*, 23, 207-213

Chassy, B. M. (1985). Prospects for improving economically significant *Lactobacillus* strains by "genetic technology". *Trends in Biotechnology*, 3, 273-275.

Chassy, B. M., & Flickinger, J. L. (1987). Transformation of *Lactobacillus casei* by electroporation. *FEMS Microbiol. Lett.*, 44, 173-177.

Chopin, M.C., Chopin, A., Rouault, A., & Galleron, N., (1989). Insertion and amplification of foreign genes in the *Lactococcus lactis* subs. *lactis* chromosome. *Appl. Environm. Microbiol.* 55, 1769-1774.

Christiaens, H., Leer, R.J., Pouwels, P.H., & Verstraete, W. (1992). Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* using a direct plate assay. *Appl. Environ. Microbiol.*, 58, 3792-3798.

Claassen E., Gerritse K., Laman J.D., Boersma W.J.A. (1991) New immuno-enzyme-cytochemical stainings for the *in situ* detection of epitope specificity and isotype of antibody forming B-cells in experimental and natural (auto) immune responses in animals and man: A review. J. Immunol. Methods, 150, 207-216.

Claassen E., Posno M. and Boersma W.J.A. (1993a) New and safe "oral" live vaccines based on *Lactobacillus*. in: Advances in Mucosal Immunology, Adv. Exp. Med. & Biology (eds. Brandtzaeg, McGhee, Mestecky, Sterzl & Tlaskalova) Plenum press, New York: in press.

Claassen I., Osterhaus A., Boersma W.J.A., Schellekens M.M. and Claassen E. (1993b) Fluorescent labeling of virus, bacteriae and iscoms in vivo : systemic and mucosal localisation patterns. in: Advances in Mucosal Immunology, Adv. Exp. Med. & Biology (eds. Brandtzaeg, McGhee, Mestecky, Sterzl & Tlaskalova) Plenum press, New York: in press.

Claassen E., M.J. Kottenhagen, P.H. Pouwels, M. Posno, W.J.A. Boersma and C.J. Lucas (1993c) The use of *Lactobacillus*, a GRAS (generally regarded as safe) organism, as a base for a new generation of "oral" live vaccines. In: "Recombinant and Synthetic Vaccines" eds. G.P. Talwar and V.S.R. Kanury. Narosa Publ. New Delhi, 405-410.

Cocconcelli, P. S., Gasson, M. J., Morelli, L., & Bottazzi, V. (1991). Single-stranded DNA plasmid, vector construction and cloning of *Bacillus stearothermophilus* a-amylase in *Lactobacillus*. *Research in Microbiol.*, 142(6), 643-652.

Fernandes, C. F., Shahani, K. M., & Ames, M. A. (1987). Therapeutic role of dietary lactobacilli and lactobacillic fermented dairy products. *FEMS Microbiol. Rev.*, 46, 343-356.

Fischetti, V.A. (1993). Streptococcal M protein. *Scientific American*, special issue, 4, 134-141.

Gerritse, K., Posno, M., Schellekens, M. M., Boersma, W. J. A., & Claassen E. (1991a). Oral administration of TNP-*Lactobacillus* conjugates in mice: A model for evaluation of mucosal and systemic immune responses and memory elicited by transformed lactobacilli. *Res. Microbiol.*, 141, 955-962.

Gerritse, K., Posno, M., Schellekens, M. M., Boersma, W. J. A., & Claassen E. (1991b). Mucosal immune response and systemic immunological memory after oral administration of TNP-*Lactobacillus* conjugates in mice. Lymphatic Tissues and In Vivo Immune Responses. *Exp. Med. & Biol.* 84, 497-504.

Gilliland, S. E. (1990). Health and nutritional benefits from lactic acid bacteria. *FEMS Microbiol. Rev.*, 87(1-2), 175-188.

Graves, M.C., & Rabinowitz, J.C. (1986). *In vivo* and *in vitro* transcription of the *Clostridium pasteurianicum* ferredoxin gene. *J. Biol. Chem.*, 261, 11409-11415.

Gruss, A., & Ehrlich, D. (1989). The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiol. Rev.*, 53, 231-241.

Gryczan, T.J., Hahn, J., Contente, S., & Dubnau, D. (1982). Replication and incompatibility properties of plasmid pE194 in *Bacillus subtilis*. *J. Bacteriol.*, 152, 722-35.

Gurr, M. I. (1987). Nutritional aspects of fermented milk products. *FEMS Microbiology Reviews*, 46, 337-342.

Holck, A., & Naes, H. (1992). Cloning, sequencing and expression of the gene encoding the cell-envelope-associated proteinase from *Lactobacillus paracasei* subsp. *paracasei* NCDO 151. *J. Gen. Microbiol.*, 138, 1353-1364.

Jones, S. & Warner, P.J. (1990). Cloning and expression of alpha-amylase from *Bacillus amyloliquefaciens* in a stable plasmid vector in *Lactobacillus plantarum*. *Lett. Appl. Microbiol.*, 11, 214-219.

Kok, J., Leenhouts, K., Haandrikman, A., Ledeboer, M., & Venema, G. (1988). Nucleotide sequence of the cell wall proteinase gene of *Streptococcus cremoris* Wg2. *Appl. Environ. Microbiol.*, 54, 231-238.

Leenhouts, K.J., Gietema, J., Kok, J., & Venema, G., (1991). Chromosomal stabilization of the proteinase genes in *Lactococcus lactis Appl. Environm. Microbiol.* 56, 2568-2575.

Leer, R., Christiaens, H., Peters, L., Posno, M., & Pouwels, P. H. (1993). Gene-disruption in *Lactobacillus plantarum* strain 80 by site-specific recombination: isolation of a mutant strain deficient in conjugated bile salt hydrolase activity. *Mol. Gen. Genet.*, 239, 269-272.

Leer, R. J., Van Luijk, N., Posno, M., & Pouwels, P. H. (1992). Structural and functional analysis of two cryptic plasmids from Lactobacillus pentosus MD353 and Lactobacillus plantarun ATCC 8014. *Mol. Gen. Genet.*, 234, 265-274.

Lerch, H-P, Frank, R., & Collins, J. (1989). Cloning, sequencing and expression of the L-2hydroxyisocaproate dehydrogenase-encoding gene of *Lactobacillus confusus* in *Escherichia coli*. *Gene* 78, 47-57.

Lokman, B.C., van Santen, P., Verdoes, J.C., Kruse, J., Leer, R.J., Posno, M., Pouwels, P.H. (1991). Organization and characterization of three genes involved in D-xylose catabolism in *Lactobacillus pentosus*. *Mol. Gen. Genet.* 230, 161-169.

Luchansky, J. B., Muriana, P. M., & Klaenhammer, T. R. (1988). Application of electroporation for transfer of plasmid DNA to *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Bacillus*. *Staphylococcus*, *Enterococcus* and *Propionibacterium*. *Mol. Microbiol.*, 2, 637-646.

Maguin, E., Duwat, P., Hege, , Ehrlich, D., & Gruss, A. (1992). New thermosensitive plasmid for gram-positive bacteria. *J. Bacteriol.* 174, 5633-5638.

McGhee J.R., Mestecky J., Dertzbaugh M.T., Eldridge J.H., Hirasawa M. and Kiyono H. (1992) The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine*, 10, 75-88.

Mercenier, A., Pouwels, P.H., & Chassy, B.M. (1993). In: Applied genetics of lactic acid bacteria. Eds. M. Gasson and W.M. de Vos. Chapman and Hall, Londen, in press.

Mestecky J. (1987) The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J. Clin. Immunol.*, 7, 265-276.

Metchnikoff, E. (1908). The Prolongation of Life. *G.P. Putnam's & Sons, New York.*
Moran Jr., C.P., Lang, N., LeGrice, S.F.J., Lee, G., Stephens, M., Sonenshein, A.L., Pero, J., & Losick, R. (1982). Nucleotide sequences that signal initiation of transcription & translation in *Bacillus subtilis*. *Mol. Gen. Genet.*,186,339-346.

O'Sullivan, D. J. & Klaenhammer, T. R. (1993). High and Low Copy Number *Lactococcus* Shuttle Cloning Vectors With Features For Clone Selection. Submitted.

Posno, M., Heuvelmans, P. T. H. M., van Giezen, M. J. F., Leer, R. J., & Pouwels, P. H. (1991a). Complementation of the inability of *Lactobacillus* strains to utilize D-xylose with D-xylose catabolism-encoding genes of *Lactobacillus pentosus*. *Appl. environ. Microbiol.*, 57, 2764-2766.

Posno, M., Leer, R. J., VanLuijk, N., VanGeizen, M. J. F., & Heuvelmans, P. T. H. M. (1991b). Incompatibility of *Lactobacillus* vectors with replicons derived from small cryptic *Lactobacillus* plasmids and segregational instability of the introduced vectors. *Appl. Environ. Microbiol.*, 57(6), 1822-1828.

Pouwels, P.H., & Leer, R.J. (1993). Genetics of lactobacilli. *Antonie van Leeuwenhoek*, submitted.

Rose, A. H. (1982). History and scientific basis of microbial activity in fermented foods, in Fermented Foods (Rose, A. H., Ed.), pp. 1-13. *Academic Press, New York.*

Scheirlinck, T., Mahillon, J., Joos, H., Dhaese, P., & Michiels, F. (1989). Integration and expression of a-amylase and endoglucanase genes in the *Lactobacillus plantarum* chromosome. *Appl. Environ. Microbiol.*, 55(9), 2130-2137.

Scheirlinck, T., de Meutter, J., Arnaut, G., Joos, H., Claeyssens, M., & Michiels, F. (1990). Cloning and expression of cellulase and xylanase genes in *Lactobacillus plantarum*. *Appl. Microbiol. Biotechnol.*, 33, 534-541.

Shimizu-Kadota, M., Shibahara-Sone, H., & Ishiwa, H. (1991). Shuttle plasmid vectors for *Lactobacillus casei* and *Escherichia coli* with a minus origin. *Appl. Environ.Microbiol.*, 57, 3292-3300.

Swinfield, M. J., Janniere, L., Ehrlich, S. D., & Minton, N. P. (1991). Characterization of a region of the *Enterococcus faecalis* plasmid pAMß1 which enhances the segregational stability of pAMß1-derived cloning vectors in *Bacillus subtilis*. *Plasmid*, 26, 209-221.

Vidgrén, G., Palva, I., Pakkanen, R., Lounatmaa, K., & Palva, A. (1992). S-layer of *Lactobacillus brevis*: PCR cloning and determination of the nucleotide sequence. *J. Bacteriol.*, 174, 7419-7427.

C.S. Vissinga, C. Dirven, F. Steinmeier, R. Benner, W.J.A. Boersma (1987) The deterioration of cellular immunity during aging: the relationship between age dependent IL-2 production ability and the frequency of Thy-1+Lyt-2-cells in C57bl/ka and CBA/Rij mice. Cell. Immunol. 108, 323-334.

Van der Heijden P.J., *Doctoral Thesis*, Erasmus University of Rotterdam (1990)

Vogel, R.F., Gaier, W., & Hammes, W.P. (1990). Expression of the lipase gene from *Staphylococcus hyicus* in *Lactobacillus curvatus* Le2-c. *FEMS Microbiol. Lett.*, 69, 289-292.

Zegers N.D., Van Holten C., Claassen E. and Boersma W.J.A. (1992) Peptide induced memory (IgG) response, crossreactive with native proteins, requires covalent linkage of a specific B cell epitope with a T cell epitope. Eur. J. Immunol., 23, 630-634.